RICE UNIVERSITY

Potential Application of D-Amino Acids in Biofouling Control of Nanofiltration (NF) Membranes

by

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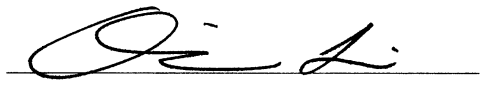
A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Master of Science

APPROVED, THESIS COMMITTEE:



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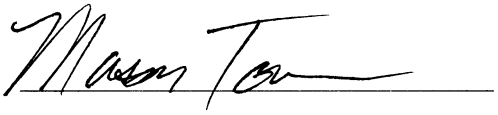

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HOUSTON, TEXAS

AUGUST 2011

ABSTRACT

Potential Application of D-Amino Acids in Biofouling Control of Nanofiltration (NF) Membranes

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Biofouling is a major impediment for the application of reverse osmosis filtration and nanofiltration in water and wastewater treatment as well as seawater desalination. In this study, a novel biofouling control strategy of using D-amino acids to interfere with biofilm formation was evaluated. Impact of D-amino acids on the surface attachment and biofilm formation of *Pseudomonas aeruginosa* was investigated in batch and continuous flow filtration systems. All 19 D-amino acids demonstrated inhibitive effects on *P. aeruginosa* biofilm formation. In particular, D-tyrosine was found to strongly inhibit *P. aeruginosa* attachment and biofilm formation on an NF membrane. When continuously supplemented to the membrane feed water in a bench scale nanofiltration system, it prohibited irreversible biofouling of the NF membrane at concentrations as low as 3 μM. The effectiveness of biofilm control by these D-amino acids seems to strongly depend on the ratio of D-amino acid concentration to bacterial cell number.

ACKNOWLEDGEMENTS

I appreciate the intellectual input and directions from my advisor, Dr. Qilin Li. It was also a great honor to have Dr. Alvarez and Dr. Tomson on my committee. I am grateful for the support from my colleagues from the lab, Xiaolei Qu, Jinjian Wu, Alison Contreras, Mike Liga, Sen Wang and Jianwei Ma. My friend, Siqi Zhao has been encouraging me through the painful process of thesis revision. Without their help, I couldn't have survived it.

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1 Introduction

Nanofiltration (NF) and reverse osmosis (RO) membranes are widely used in water and wastewater treatment and seawater and brackish water desalination. Wastewater reclamation and seawater desalination are important supplement to alleviate the demand for freshwater resource. The undesirable attachment of microbes and development of biofilm on wet surface are considered as biofouling. Biofouling is a major impediment in water purification and reuse applications of NF and RO membranes. It not only causes permeate flux decline and membrane performance deterioration, such as high salt passage, but also gradually degrades membrane material. Despite the many different approaches that have been taken, prevention and control of biofouling remains unarguably the greatest barrier of applying membrane technology in water and wastewater treatment and seawater desalination. Current anti-biofouling strategies include pretreatment (e.g. coagulation/flocculation, biofiltration, MF/UF filtration and UV irradiation(*1*)) of feed water to remove organics and microorganisms, addition of antimicrobial reagents, modification of membrane surface properties (e.g., hydrophobicity, roughness and charge) to reduce bacterial cell attachment, optimization of operation conditions (e.g., pH, pressure, and

flux), and post membrane cleaning. Pretreatment of feed water usually cannot remove microorganisms completely, and even a small number of surviving microorganisms could attach to and multiply on the membrane surface causing severe biofouling. Chlorination is typically used in surface water treatment plants to inhibit microbial growth in feed water. 0.5 ppm residue chlorine is often required for microbial control. However, such concentration is usually too high for polyamide based NF and RO membranes. Oxidation by chlorine can damage the membrane and lead to loss of salt rejection and removal of other contaminants. Therefore, sodium bisulfite (SBS) is usually needed to quench chlorine prior to NF/RO membrane units. Chlorine also breaks down large organic molecules into more readily biodegradable small molecules that encourage bacterial growth and leads to high concentration of disinfection byproduct formation. Therefore, the surviving bacteria will thrive. Frequent membrane cleaning could reduce lifespan of NF/RO membranes. None of these existing methods is very effective in controlling biofouling. High efficiency, environmental friendly and low-cost strategies of biofouling control are greatly needed.

The novel approach of using biological control to mitigate biofouling in membrane filtration systems has been drawing increasing attention recently. Biological control strategies include interfering bacterial quorum sensing,

using messenger molecules such as nitric oxide (NO) to disperse biofilms, adding bacteria phage and enzyme hydrolysis of cell wall etc. (*1-3*) Quorum quenching and messenger molecules do not inactivate bacteria but can prevent or disrupt biofilm, which is often desired in wastewater treatment plants to avoid biomass loss and disturbance of the biological treatment process. Both approaches have been tested in the lab and shown biofilm inhibition or disassembly effect in a wide range of bacteria.(*3*) However, their effectiveness in membrane systems in practice is unknown.

Recent scientific discoveries suggest that D-amino acids may be used for biofouling control. D-amino acids are produced by many bacteria in their cell walls.(7) Ilana Kalodkin-Gal *et al.* found that the D-amino acids (D-tyrosine, D-methionine, D-tryptophan and D-leucine) produced by *Bacillus subtilis* can prevent the formation and trigger the disassembly of *B. subtilis* biofilm at liquid-air interfaces(*6*); D-tyrosine also inhibited biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* on polystyrene plate surface.(5) The effective concentration of D-tyrosine preventing *B. subtilis* biofilm formation was as low as 3 μM. (*7*) Interestingly, these D-amino acids did not inhibit bacteria growth.(*6*) Since D-amino acids are stable in natural and waste water conditions and are not expected to damage

membrane materials, they could potentially be used in membrane systems to control biofouling.

The objective of this research is to assess the effectiveness of D-amino acids in inhibiting biofilm formation on nanofiltration membranes and further preventing biofouling in NF systems. D-tyrosine, which was shown to be highly effective in inhibiting *B. subtilis* biofilms, was chosen as the model D-amino acid. A well-known biofilm forming bacterium, *P. aeruginosa*, was used in all experiments. Attachment of *P. aeruginosa* on a commercial NF membrane was measured in LB media with and without D-tyrosine treatment. The biofouling control ability of D-tyrosine was then examined in a bench-scale NF system by continuously adding D-tyrosine into two different feed waters. Finally, 19 D-amino acids were screened for biofilm inhibition potential using the crystal violet biofilm assay.

2 Membrane and water quality

Water is essential for the survival of human being, and it is a critical issue for areas lacking adequate freshwater resource or proper techniques to obtain safe drinking water. Wastewater reclamation is considered an important supplement for freshwater.(*8*) Filtration, the process of removing undesired constituents from aqueous or gas phase, has long been used as water purification method, and sand or sieves are common separators. In traditional filtration systems (sieves or sand), fluid is often driven by gravity. In many parts of the world, membranes are replacing conventional media filters to improve water quality. Depending on the type used, membranes can remove particulate matter, colloidal materials, dissolved organic matter and even salt ions.

A membrane is a thin layer of semi-permeable material that performs phase separation based on their physical or chemical properties. Many kinds of synthetic materials can be used for preparing membranes such as ceramics, glass, metal, or polymers. In water purification, polymer and ceramic membranes are often used. Pressure driven membranes are classified by their selectivity and pore sizes into microfiltration (MF), ultrafiltration (UF), nanofiltraion (NF) and reverse osmosis (RO) membranes. The first three kinds of membranes are porous membranes, which separate different

materials mainly based on size exclusion. NF membranes also reject salt by Donnan exclusion. MF membranes are capable of removing suspended solids including bacterial and fungal cells; UF membranes are capable of removing fine particles including viruses, colloidal materials and macromolecules; NF membranes are capable of removing multivalent ions, some pesticides, herbicides and endotoxins. NF membranes are mostly used in surface water or brackish groundwater treatment. More recently, they are being considered for wastewater treatment.(*9*) RO membranes, on the other hand, are non-porous and the salt separation mechanism is Donnan exclusion. RO membranes are capable of removing single valent ions and are often used in desalination or wastewater reuse. NF and RO membranes are mostly polyamide (PA) thin film composite (TFC) membranes synthesized by interfacial polymerization method on microporous polysulfone supports or asymmetric cellulose acetate (CA) membranes synthesized by phase inversion on porous woven polyethersulfone fabric. (*10*) The driving force of NF and RO membrane filtration is pressure. The higher the rejection, the more pressure or energy is needed. Because of their high productivity and selectivity, membranes are becoming increasingly important in drinking water and wastewater treatment processes.

3 NF/RO membrane biofouling

3.1 Mechanisms and consequences

Membrane fouling originates from particulate matter, colloids, dissolved organic matter, precipitation of salts (scaling), and microorganisms in the feed water. Among these, fouling from microorganisms i.e. biofouling, is the most difficult to control.(*11*) It has been reported that biological foulants account for 50% dry weight of total foulants on NF/RO membranes in wastewater and surface water treatment plant.(*12*)

Biofouling is caused by deposition, attachment and multiplication of microorganisms on the membrane surface. The biofouling process is usually considered to consist of 4 stages. It starts with deposition of bacterial cells on the membrane, which occurs in seconds (*13*). This is followed by irreversible attachment of cells in seconds to minutes. In the third stage, bacterial cells grow, produce extracellular polysaccharides substances (EPS) and develop into a biofilm.(*14*) Finally, in response to limited nutrients and accumulating toxins, the biofilm will eventually degrade and disassemble. In the first stage, physicochemical interactions between the membrane surface and bacterial cell surface play an important role in regulating cell deposition rate. Therefore, changing of membrane surface properties including surface

charge and hydrophobicity could affect cell deposition. Hydrophobic, non-polar surface is normally more favorable for cell attachment to happen. (*13*, *15*) The motility of bacteria cell has also been shown to be important in cell attachment and biofilm formation.(*16*) It is reported that the *P. aeruginosa* phenotype that lacks motility forms defective biofilm on PVC surfaces.(*17*) It is considered that the flagella motility of bacteria is useful in overcoming the electrostatic repulsion between the membrane surface and bacteria.(*18*) Besides membrane surface properties, solution conditions (i.e. pH, ion species and ionic strength) and operation conditions (*19*) (i.e. flow rate and shear force on membrane surface) also affect cell deposition. After deposition, bacteria produce extracellular polymeric substances (EPS) that allows cell attachment to happen, which is necessary for biofilm to develop. (*20*) EPS is the complex surrounding microbial cells that usually consists of proteins, carbohydrates, acid polysaccharides, DNA, lipids, and humic substances. (*21*, *22*) The bacteria can not only attach to membrane surface, but also to other cells, which leads to multilayer biofilm formation. After attachment, the bacteria reproduce and develop microcolonies and then mature biofilm. The development of biofilm is regulated by various signals from the environment (e.g. nutrient level, osmotic pressure, mechanical forces, antimicrobials, etc.) and from cells (quorum sensing).(*23*) With the

protection of biofilm, the bacteria can better adapt to various environments. The biofilm enables resistance of bacteria towards shear force, biocidical compounds and predator in feed water.(*24*)

Impacts of fouling on NF and RO membranes include deterioration in membrane performance (i.e. high salt passage and flux decline) and degradation of membrane material. Such impacts lead to higher energy and maintenance costs and shorter lifespan of membranes and membrane modules.(*10*) Two mechanisms of biofouling affecting NF/RO membrane performance have been identified previously: 1) The biofilm adds to the hydraulic resistance of cake layer. 2) The biofilm hinders the back diffusion of salt ions, leading to increased salt concentration at the membrane surface. EPS produced by bacterial cells plays a dominant role in building up hydraulic resistance on the membrane surface, decreasing permeate flux (or increasing transmembrane osmotic pressure). (*25*, *26*) The increased salt concentration results in higher osmotic pressure (*16*), which enhances concentration polarization, leading to flux decline. The higher salt concentrations at the membrane surface also deteriorate membrane performance by increasing salt passage. According to Herzberg *et al.,* the increased concentration polarization mechanism contributes more to flux decline than increased hydraulic resistance.(*16*) In spiral-wound membrane

modules, biofouling can block channels of flow and cause flux decline.(*27*) In hollow fiber systems, individual fibers can be bound together by foulants (especially by EPS), which results in difficulties for cleaning agents to reach each fiber thus the membrane is not effectively cleaned.(*28*)

3.2 Anti-biofouling strategies in NF/RO filtration

Despite the vast efforts that have been taken, biofouling remains the untackled problem in NF/RO membrane filtration systems. It is difficult to control for the following reasons. Firstly, pretreatments of feed water are not as effective when targeting biofouling although other types of fouling can be largely reduced. Particulate matter and colloids can be removed by coagulation and flocculation treatments or MF/UF membranes. Pretreatments for scaling include adjusting pH of feed water and addition of anti-scalants. For organic fouling, UF membrane filtration, activated carbon adsorption and hydroxide coagulation are common pretreatments to remove the foulants from feed water (*1*). Bacteria are like colloids in size but they can reproduce. Thus, a small amount of bacterial cells can develop into severe biofouling problem in non-sterile systems. Even 99.99% removal of microbes in feed water cannot prevent eventual biofilm formation on membrane surface. (*14*, *29*) It is unrealistic to remove the entire bacteria population from feed water. The bacterial growth relies on the nutrients in

feed water. In water treatment, there are usually abundant natural organic matters (NOM) in surface water and ground water for cell growth, which are the common feed of drinking water treatment plants.(*30*) In wastewater, organics are even more abundant from the municipal waste and dead cell debris. Moreover, due to rejection of the organics by NF/RO membranes, nutrients accumulate on membrane surface. Thus even if the organics and cell debris are reduced with pretreatment that the nutrient level in feed water is low, the bacteria attached to membrane surface can still survive. Because of the fouling from organics, it is more favorable for bacteria to grow on surface than as planktonic cells. (*29*) Furthermore, biofouling is difficult to clean since bacteria are embedded in EPS, strongly adhering to membrane surface and protecting the cells from biocides.(*13*) Therefore, biofouling is considered as the Achilles heel of membrane processes. (*11*)

To reduce biofouling, there are several available approaches: pretreatment to remove nutrients and microbes from feed water; addition of antimicrobial reagents into feed water or membranes; modification of membrane surface to reduce cell attachment; optimization of operation conditions (hydrodynamic and solution conditions); membrane cleaning; and biological control.

3.2.1 Pretreatment of feed water

To reduce biofouling on NF and RO membranes, pretreatments of feed water to remove colloids and organics are necessary since they can induce biofouling on membranes.(*31*) Conventional pretreatment for seawater desalination is a train of coagulation flocculation, air flotation and granular media filtration. Silt density index (SDI) is a commonly measured factor that relates to fouling potential of feed water and SDI smaller than 5 indicates low biofouling potential. (*32*) In modern desalination plants, MF/UF membrane treatment of feed water is preferred to reduce SDI. In a pilot trial in Singapore, K.T. Chua *et al.* found that MF/UF treatment constantly kept the feed water SDI below 3.0 while conventional treatment frequently peaked at 6.3, which was much higher than the prerequisite level for RO membrane (SDI $\leq$ 5.0). (*33*) Removal of assimilable organic carbon (AOC) and biodegradable dissolved organic carbon (BDOC), carbon and energy sources for bacteria, was proved to reduce biofouling of RO membranes significantly.(*34*, *35*) Biofiltration was found to be an effective and economic pretreatment for AOC reduction in feed water to control biofouling on RO membrane.(*36*)

3.2.2 Addition of antimicrobial materials

Chemical disinfectants, such as chlorine and ozone, can be added to membrane feed water in order to control microbial concentration and biofouling in membrane filtration systems. However, these disinfectants are also strong oxidants. Most thin film composite NF and RO membranes are made of polyamide, which cannot stand strong oxidants. As is suggested by the manufacturer, the highest free chlorine tolerance of NF 270 membrane is 0.1 ppm in feed water. (*1*, *24*) Therefore, free chlorine and ozone must be removed from feed water prior to enter RO/NF membrane unit by addition of sodium bisulfite (SBS) or by activated carbon adsorption. (*1*) Another problem related to the use of chlorine or ozone is the degradation of large organic molecules into lower molecular weight, more readily biodegradable molecules, which encourage bacterial growth. Outburst of bacteria growth after removing chlorine residue from feed water was observed due to the formation of AOCs and the absence of biocide.(*34*, *37*) Therefore, less potent chlorine compounds, monochloramine and chlorine dioxide, are sometimes used in the place of free chlorine. However, with any chlorine disinfectant, formation of disinfection byproducts is of concern. Ozone can also form carcinogenic bromate.(32)

3.2.3 Membrane cleaning

In practice, NF membranes need to be cleaned when the permeate flux decline to 85% of initial flux or when pressure increases by 15% of the initial pressure. (*12*) Chemical cleaning is usually required to achieve flux recovery. A low pH solution is used to remove inorganic scaling and a high pH solution is used to remove organic foulants.(*39*) To remove specific foulants, chelating reagents, surfactants, enzymes (*40*), and oxidants are often used as cleaning chemicals between the acid and alkaline washing.(*41*) Enzyme cleaning of biofouling is superior to oxidation compounds because it does not degrade membrane material but can target specific compounds in EPS. However, since enzymes only interact exclusively with certain substrates, it would be difficult to select an enzyme to target the whole broad microbial spectrum in the biofilm.(*40*) Also, the process of enzymatic degradation of EPS takes long time to occur(*3*). Therefore, it is desirable to have a universal and effective cleaning agent that does not degrade membrane material.

3.2.4 Operation condition optimization

Biofouling can be controlled by modification of hydrodynamic conditions. In cross-flow systems, permeate convective flow is closely related to cell deposition on membrane surfaces. It has long been assumed that below a

critical flux, no colloidal attachment would happen in a cross-flow system (*42*) or no flux decline would occur with time.(*43*) The critical flux concept was first established for particulate or colloidal fouling of MF or UF membranes. The bacterial cells and cell debris have approximately the same sizes as suspended particles and colloidal materials, which indicates that there could also be a critical flux for biofouling on NF and RO membranes. However, J. Vrouwenvelde *et al.* found that flux decline with time in NF and RO spiral wound systems caused by biofouling could not be prevented by operating below a "critical flux" since the nutrients and microbial cells are rejected by the membrane and accumulate at the feed side of membrane surface creating favorable conditions for biofilm growth. (*44*)

Besides hydraulic conditions, operation temperature also affects biofouling potential. Higher operation temperature (>25 °C) is more favorable for bacteria growth hence promoting biofouling.(*32*, *45*) pH and ionic strength of feed water are also important by impacting electrical double layer of membrane and foulants in solution. It has been reported that Ca^{2+} could bridge the major EPS component alginate in *P. aeruginosa* biofilm and escalate biofouling in membrane systems. (*46*, *47*)

3.2.5 Membrane surface modification

Two approaches of improving membrane biofouling control by surface modification include anti-adhesion and anti-bacteria. The adhesion of microbes on membrane surface depends on membrane and cell surface properties, such as hydrophobicity, roughness and charge.(*48*) In natural conditions, most bacteria are negatively charged and hydrophobic. It is observed that hydrophilic membrane surface is less susceptible to hydrophobic cell adhesion. (*49*, *50*) It has been reported that though attractive for negatively charged bacteria, positively charged membrane surfaces tend to cease bacterial growth due to the high electrostatic force.(*51*) Membrane surface roughness was suggested to be the most important factor affecting cell attachment.(*52*) Bacteria can be considered as colloids and colloidal foulants tend to accumulate in valleys on membrane surface first which lead to flux decline. (*53-55*) The valleys are also favorable for organic foulants to attach, which creates suitable conditions for bacteria to grow. (*31*) The rough surfaces increase difficulties in cleaning as well. However, though initial adhesion can be reduced, the impact of the surface properties on biofilm development is not significant, which is the limitation of this approach. (*50*) Grafting monomers on membrane surface to increase hydrophilicity have shown to reduce attachment of organic and biological

fouling.(*56*) Though increasing hydrophilicity, polymer with surface charge or hydrogen bonding sites were found to increase adhesion of biopolymers also.(*57*) Grafting of zwitterionic materials and hydrogen bond acceptors was studied instead and shown anti-biofouling effect. (*58*)

Biocides can be incorporated into membrane material to prevent biofouling. Silver ion or nano silver are widely studied antimicrobial reagents.(*59*) Nano silver was immobilized onto polyamide membrane surface and spacer to effectively prevent biofouling by reducing cell adhesion and inactivating the attached cells. (*60*, *61*) Zhu *et al.* immobilized ionic and reduced silver into chitosan membranes, which showed inhibition of bacteria adhesion and biofouling. (*62*) Zodrow *et al.* blended nanosilver into polysulfone UF membranes and effectively inactivated *E. coli* and *P. aeruginosa* as well as MS2 phage. However, the fast leaching of silver on surface remained a problem. (*63*) Besides silver, TiO_2 was also used as anti-biofouling agent incorporated into hybrid polyamide RO membranes due to its photocatalytic ability. (*64*, *65*)

3.2.6 Biological control of biofilm

Biological control of biofilm is an innovative approach for biofouling control. Instead of inactivating the microbes, some biological control methods only interfere with the biofilm forming process, including

interference with bacterial quorum sensing, utilizing signal molecules such as NO. (*3*, *23*) Therefore such methods are useful in membrane bioreactors, where bacterial death is undesirable.

In quorum sensing, bacteria produce autoinducers (AIs) for intercellular communication. When reaching certain concentration levels, the autoinducers can trigger a series of group activities in a bacteria community, such as biofilm formation, biofilm dispersal, antibiotics production, motility, swarming, EPS production etc. (3-6) The method of mitigating bacteria behavior by inhibiting the AIs in quorum sensing is called quorum quenching.

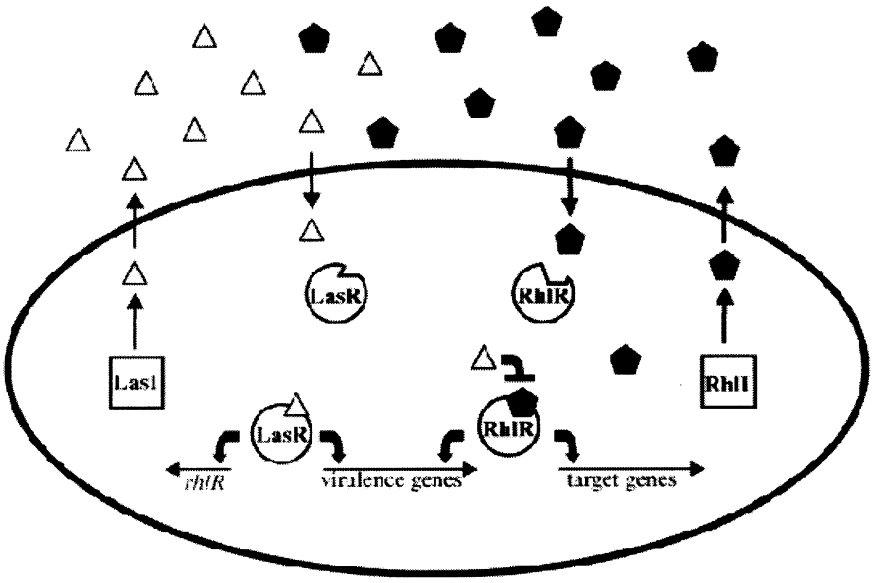

Figure 3.1 Quorum sensing system of *P. aeruginosa*.(*68*)

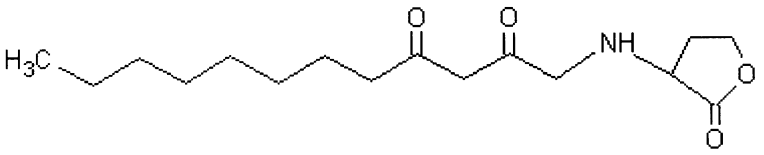

Figure 3.2 Structure of *P. aeruginosa* LasI directed AI N-(3-oxododecanoyl)-L-homoserine (3OC12HSL)

Three types of autoinducers have been discovered including oligopeptides (works in gram-positive bacteria), N-acylhomoserine lactones (AHL, works in gram-negative bacteria) and autoinducer-2 (AI-2, works in both gram-negative and gram-positive bacteria). (*69*) These AIs are closely related to bacteria cell attachment on surface and biofilm development. For instance, *P. aeruginosa* has LasI/LasR and RhlI/RhlR quorum sensing systems that produce extracellular AIs (N-(3-oxododecanoyl)-L-homoserine lactone as triangles and N-buytryl-L-homoserine lactone as pentagons in Figure 3.1). When AIs produced by LasI and RhlI encoded proteins reach critical concentration, they bind to LasR and RhlR receptors to trigger virulence genes expression or secondary quorum sensing system. (*68*) According to the study of Davis *et al.*, LasI produced AIs (structure shown in Figure 3.2) were essential in biofilm differential but not in the initial attachment stage.(*70*) Comparing to wild type, mutants of *P. aeruginosa* without LasI produced AIs developed thinner biofilm with denser cell concentration and the distribution of glycocalyx was close to planktonic cell.(*70*) Therefore, quenching the signaling process of these 3 AIs could lead to inhibition of

biofilm formation. Barrios *et al.* found that addition of AI-2 increased *E. coli* biofilm formation by enhance motility of *E. coli,* and AI-2 was synthesized by LuxS synthase. (*71*)Therefore, analogs of LuxS substrate could bind with LuxS receptor and hence hinder the biofilm formation behavior expedited by AI-2.(*3*) Furanone or its derivatives were found to be effective in disrupting the biological synthesis process of AI-2 thus could be used as biofilm control agents. Analogs of furanone have been shown to cause *P. aeruginosa* biofilm detachment (a derivative of natural furanones lacking alkalyl side chain) (*72*) and inhibit attachment of *P. aeruginosa* on glass slide ((5-oxo-2,5-dihydrofuran-3-yl)methyl alkanoate) (*73*). AHL inhibitors, such as vanillin extracted from vanilla beans, cyclic sulfur compounds from garlic, halogenated furanone from *Delisea pulchra* and patulin and penicillic acid from fungi, (have similar structures as AHL AIs that are able to bind with receptors but cannot function as AIs) were reported to effectively mitigate biofilm formation by interacting with AHL receptors. (*73*, *74*) AHL-degrading enzyme Acylase I was also found to inhibit *A. hydrophila* and *Pseudomonas putida* biofilms on polystyrene and RO membrane surfaces by cleaving amide groups on AHL.(*75*)

Nitric oxide (NO) is a signal molecule in bacterial cell-to-cell communication that regulates the disassembly of biofilm. (*3*) Barraud *et al.*

found that addition of 500 nM (non-lethal level) NO donor sodium nitroprusside (SNP) caused the dispersal of *P. aeruginosa* biofilm on glass slide and enhanced the biofilm removal efficacy of antimicrobial agents. (*76*) The same study also demonstrated that the biofilm of a mutant (Δ*nirS*) unable to produce NO failed to disperse while the biofilm of a mutant (Δ*norCB*) producing a large amount of NO was easily dispersed.(*76*)

The use of quorum quenchers or signal molecules for biofilm control does not kill bacteria but triggers the dispersal or regulates the development of biofilm. Thus the likelihood for bacteria to develop resistance to these methods is low. The chemicals used are nontoxic. Therefore, these methods are attractive options for membrane biofouling control. Further research is needed to evaluate their feasibility and cost-effectiveness.

3.3 Potential of D-amino acids in biofouling prevention

In most forms of life, L-isomers of amino acids are preferably utilized in synthesis of proteins and biological processes compared to their D-isomers. D-amino acids are commonly found in bacteria cell wall. Lam *et al.* found that the D-amino acids in cell wall are related to peptidoglycan synthesis. (*5*)

More recently, D-amino acids were reported to prevent formation and induce disruption of bacterial biofilms without affecting bacterial growth. (*6*)

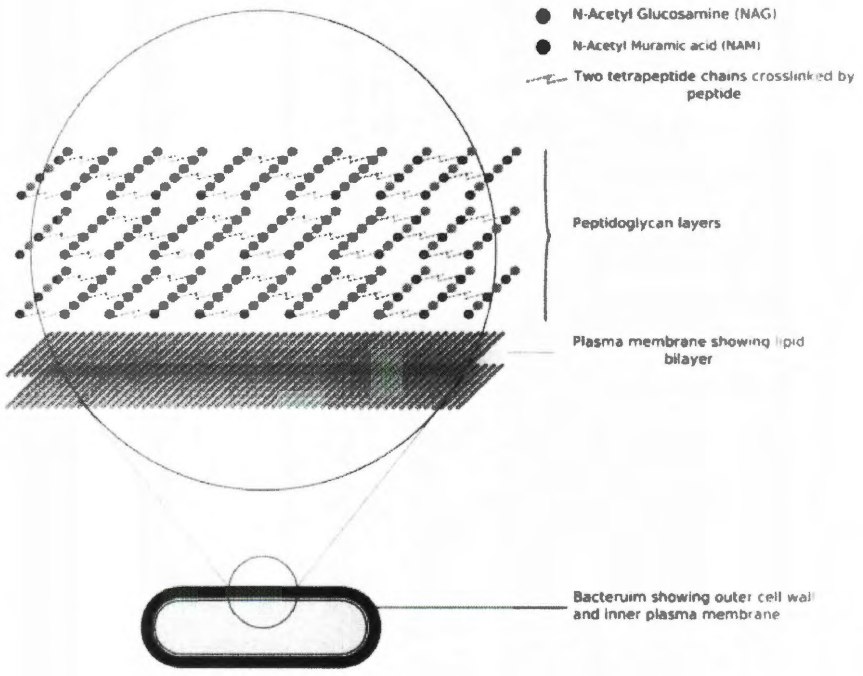

Figure 3.3 Schematic of gram-positive bacteria cell wall (*77*)

Gram-positive bacteria, such as *Bacillus subtilis*, have cell walls outside the lipid bilayer of bacterial membrane. (Structure shown in Figure 3.3) The cell wall in Gram positive bacteria consists of a thick peptidoglycan (PG) layer, which helps to maintain the osmotic pressure and membrane shape. The composition of peptidoglycan is shown in Figure 3.4, where the backbone of PG is made of polysaccharides consisting of alternating N-acetylmuramic

acid (NAM) and N-acetylglucosamine (NAG) in equal amounts. The polysaccharides backbones are cross-linked by peptide chains.

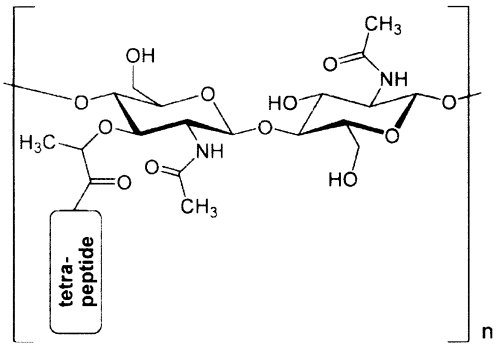

Figure 3.4 Structure of peptidoglycan(*78*)

However, the cell wall of gram-negative bacteria such as *Escherichia coli*, *P. aeruginosa* etc. are different from gram-positive bacteria, which consists of an outer membrane and a thin peptidoglycan layer. The schematic of gram-negative bacteria cell wall is shown in Figure 3.5. The outer membrane has lipopolysaccharides (LPS) attached to the lipid bilayer. LPS, which is closely related to cell-surface interaction, is claimed to play a critical role in gram-negative biofilm.(*16*, *79*) The major components of LPS (structure shown in Figure 3.6) include lipid A, core polysacharide and O-antigen. O-antigen is the hydrophilic part of LPS pointing into the environment and consists of 15 to 20 repeating subunits of three to five sugars, which are strain specific.(*80*) It is often used to distinguish different strains of bacteria. Lipid A is the hydrophobic fatty acid that anchors LPS in the lipid bilayer

and is responsible for the toxicity of gram-negative bacteria that can trigger the human immune response.(*81*) O-antigen and lipid A are connected by core polysaccharide. Amide-linked L-alanine is found to be a unique but regular component of *P. aeruginosa* core polysaccharide. (*82*)

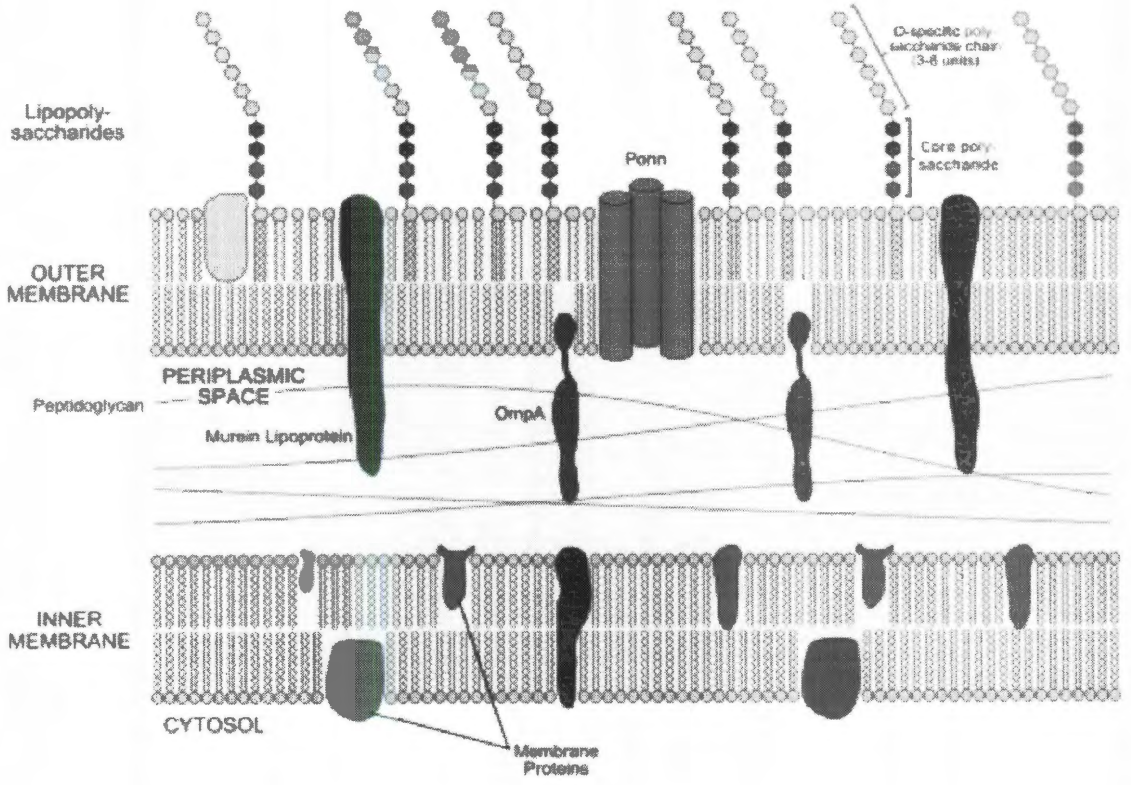

Figure 3.5 Structure of gram-negative bacteria cell wall. (*83*)

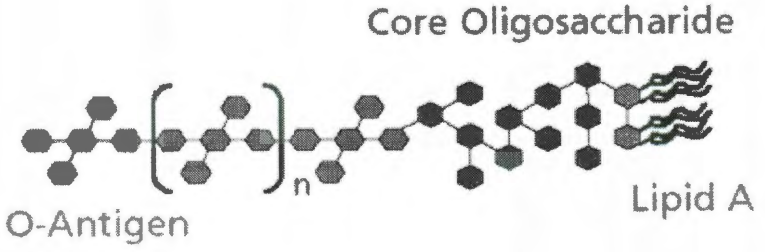

Figure 3.6 Structure of LPS. (*84*)

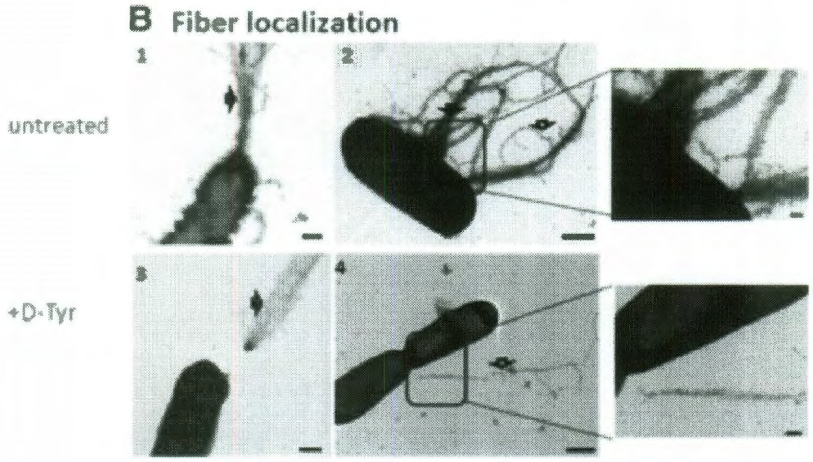

Figure 3.7 TEM images Bacillus subtilis showing detachment of TasA fibers after treatment with D-tyrosine. (*6*)

Several D-amino acids were recently found to inhibit *Bacillus subtilis* biofilm formation on liquid air surface while the L-isomers did not. (*6*) In nutrient limiting environment, forming pellicle is more beneficial than planktonic existence for bacteria. However, after 3-5 days incubation, the biofilm disassembled by itself. Kolodkin-Gal *et al.* found that such disassembly was triggered by D-amino acids produced by *B. subtilis*. They identified 4 D-amino acids (D- tyrosine, D- tryptophan, D-leucine and D-methonine) from an 8-day culture of *B. subtilis* biofilm. When added individually to a fresh inoculum in a liquid medium or on a solid surface, these D-amino acids prevented the formation of *B. subtilis biofilm* at low concentrations (3 μM, 5 mM, 8.5 mM and 2 mM for D- tyrosine, D-tryptophan, D-leucine and D-methonine, respectively). A synergistic effect was found when all 4 of these D-amino acids were added together; the

effective concentration was as low as 10 nM. Using ^{14}C labeled D-tyrosine, it was found that D-tyrosine was incorporated into *B. subtilis* cell wall, replacing D-alanine in the peptidoglycan layer. As a result, the TasA filber that anchored into bacteria cell wall was detached (Figure 3.7). (*6*)

In the same study, biofilm formation by *Staphyloccocus aureus* (gram positive) and *Pseudomonous aeruginosa* (gram negative) on polystyrene plates with and without D-amino acids supplement was also investigated with crystal violet assay of biofilm. D-tyrosine and mixed D-amino acids (D-tyrosine, D-methionine, D-tryptophan and D-leucine) appear to have prevented the biofilm on polystyrene surface while L-tyrosine did not have any inhibitive effect. D-alanine was also fond to counteract the effects of D-tyrosine and mixed D-amino acids. (*7*)

The results for *P. aeruginosa* are interesting because gram-negative bacteria have completely different cell walls comparing to gram-positive bacteria. The peptidoglycan layer of *P. aeruginosa* is buried inside the outer membrane. The attachment of cells cannot be attributed to TasA fiber as in the case of *B. subtilis*. A different mechanism must govern the inhibition of *P. aeruginosa* biofilm by D-amino acids.

The study by Kolodkin-Gal *et al.* tested 3 bacterial species, including both Gram positive and Gram negative bacteria.(*6*) Although it is hypothesized that D-amino acids are common signal compounds that trigger bacterial biofilm disassembly, (*5*)experiments with other bacterial species have not been reported.

The most commonly found D-amino acids in bacteria are D-alanine and D-glutamine, which are components of the peptidoglycan layer in bacteria cell wall.(*85*) Lam *et al.* found that besides D-alanine and D-glutamine, a variety of D-amino acids were produced by different kinds of bacteria. (*5*)19 D-amino acids were measured in 9 bacteria species. D-valine, D-tyrosine, D-threonine, D-phelyalanine, D-methionine, D-leucine, D-Isoleucine and D-alanine were detected at concentrations higher than 10 μM. The D-amino acids were found to affect peptidoglycan layer directly or indirectly. When exposed to D-methionine in the stationary phase, *E. coli* and *V. cholera* could incorporate D-methionine into the peptidoglycan layer, resulting in changes in the strength and flexibility of the cell wall.(*86*, *87*) Interestingly, exposure to D-alanine in the stationary phase led to a change in a *V. cholera* mutant cell shape from rod to sphere even though D-alanine was not incorporated in peptidoglycan. (*5*) It was hypothesized that D-amino acids

serve as signal molecules in bacteria cells. Therefore, Lam *et al.* also showed that bacteria could respond to exogenous D-amino acids.(*5*)

Since D-amino acids are produced widely by gram negative and gram positive bacteria and regulate biofilm assembly and disruption, novel biological control method can be developed based on the D-amino acids inhibition of biofilm formation.

4 Experimental

4.1 Materials

4.1.1Model bacteria and media

Pseudomonas aeruginosa (*P. aeruginosa*) ATCC# 700829 was used as model biofilm forming bacteria in all experiments. *P. aeruginosa* is found to contribute to biofouling on NF membranes in wastewater treatment (*29*) and is a common model bacteria for membrane biofouling studies(*16*, *25*, *88*). It is a rod shaped, Gram negative bacterium that has an outer membrane covering the PG layer. The *P. aeruginosa* cells were kept at -80°C in glycerol and retrieved onto a tryptic soy agar (TSA) plate. After incubating for 24 hr at 37°C, the retrieved culture was reinoculated onto a new TSA plate and incubated again for 24 hr. The stock bacteria were then stored in

dark at 4 °C and re-inoculated every week. Liquid cultures of *P. aeruginosa* were prepared by transferring one loopful of the bacterial colony from the stock into trypticase soy broth (TSB) and incubating at 37 °C for 24 hr, when the OD600 of the liquid culture reached ~1. The accurate cell concentration was measured by plate counting using TSA plates. The liquid culture was serially diluted with sterilized phosphate biological saline (PBS) and streaked on TSA plates, which were incubated at 37 °C for 20 hr before counting.

BBL™ Luria-Bertani (LB) Broth (BD, purchased through Fisher Scientific, Pittsburg, PA) was used as the liquid media for the attachment experiments. BBL™ Trypticase soy broth (BD) and Bacto™ Agar (BD) used to prepare TSA plates and TSB liquid culture were also purchased through Fisher Scientific, Pittsburg, PA.

4.1.2 Membrane

Since NF and RO membranes are both polyamide based thin film composite membranes and have similar applications, the fouling mechanisms and control methods are similar. A commercial TFC nanofiltration membrane NF 270 (Dow FilmTec, Minneapolis, MN) was used in all experiments. The NF 270 membrane has high rejection for organics and medium rejection for salts and hardness, which is suitable for surface water and groundwater

treatment.(*1*) The membrane was received as a dry flat sheet. Prior to use, the membrane was cut into coupons of required sizes, rinsed with and stored in ultrapure water (18.0 Ω, generated by E-pure system, Barnstead, IL) at 4 °C for at least 24 hr (water changed regularly).

4.1.3 Chemicals

Inorganic salts used in feed water including $CaCl_2$, sodium citrate dihydrate, ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$), NH_4Cl, and KH_2PO_4 were reagent grade and purchased from Fisher Scientific, Pittsburg, PA. The fluorescence dye 4', 6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich, St. Louis, MO. The crystal violet dye was purchased through Fisher Scientific, Pittsburg, PA. The ethanol (Fisher Scientific, Pittsburg, PA.) used to extract CV dye is 200 proof.

There are naturally 20 amino acids found as components of proteins, 19 of which have D-isomers and were all evaluated in biofilm prevention screening as listed in Table 4.1.

Table 4.1 19 D-amino acids.

	Molecular Weight (g/mol)	Purity	Vendor
D-Alanine	89.09	98%	Sigma-Aldrich,St. Louis, MO
D-Arginine	174.2	99%	Sigma-Aldrich,St. Louis, MO
D-Asparagine monohydrate	150.14	99%	Alfa Aesar, Wardhill, MA
D-Aspartic Acid	133.1	99%	Alfa Aesar, Wardhill, MA
D-Cysteine	121.16	99%	Sigma-Aldrich,St. Louis, MO
D-Glutamic acid	147.13	99%	Sigma-Aldrich,St. Louis, MO
D-Glutamine	146.14	99%	MP Biomedicals, Solon, OH
D-Histidine free acid	155.15	99%	MP Biomedicals, Solon, OH
D-Isoleucine	131.17	98%	Fisher Scientific, Pittsburg, PA
D-Leucine	131.17	99%	Sigma-Aldrich,St. Louis, MO
D-Tyrosine	181.19	99%	Sigma-Aldrich,St. Louis, MO
D-Methionine	149.21	99%	MP Biomedicals,Solon, OH
D-Phenylalnine	165.19	98%	Sigma-Aldrich,St. Louis, MO
D-Proline	115.13	99%	Sigma-Aldrich,St. Louis, MO
D-Serine	105.09	98%	Sigma-Aldrich,St. Louis, MO
D-Threonine	119.12	98%	Sigma-Aldrich,St. Louis, MO
D-Tryptophan	204.23	99%	Alfa Aesar, Wardhill, MA
D-Valine	117.15	98%	Sigma-Aldrich,St. Louis, MO
D-Lysine	146.19	98%	Sigma-Aldrich,St. Louis, MO

4.2 Methods

4.2.1 Bacterial attachment experiments

According to Kolodkin-Gal *et al.*, D-tyrosine had the ability to prevent *P. aeruginosa* biofilm formation on polystyrene surfaces at a concentration of 10 μM.(*7*) To determine whether D-tyrosine is able to prevent cell attachment or biofilm formation on membrane surfaces, a short-term and a long-term membrane attachment experiment were conducted.

NF 270 membrane was cut into 2 cm^2 coupons to fit in the 6-well cell culture plates and immersed in ultrapure water for 24 hr at 4 °C prior to use. To prepare *P. aeruginosa* liquid culture, 6 mL of TSB was inoculated with one loopful of *P. aeruginosa* colony from TSA plate. The liquid culture was incubated at 37 °C for 24 hr when it reached the late exponential phase. The cell concentration was 6×10^8 CFU/mL as measured by plate counting. The NF 270 membrane coupons were sterilized with 200 proof ethanol, rinsed with sterilized DI for 3 times and put into polystyrene 6-well cell culture plates.

The experimental conditions used are summarized in Table 4.2. In the short-term experiment, the liquid culture of *P. aeruginosa* was diluted 100 times with LB broth (10^6 CFU/mL) with or without D-tyrosine and incubated at

37 °C on a Innova 2000 platform shaker (New Brunswick Scientific, Edison, NJ) for 0.25, 0.5, 1hr at 70 rpm. To obtain inoculum with 3 μM and 30 μM D-tyrosine, 5 μL and 50 μL of 3 mM D-tyrosine stock solution were added to the 5 mL inoculum.

In the long-term experiment, the liquid culture of *P. aeruginosa* was diluted with LB broth to 10^5 CFU/mL. Samples were prepared in the same way as in the short-term experiment, but were incubated for 2, 4, 10, 24, and 34 hr at 37 °C (3 replicates for each condition). Because cell growth was limited during first hour of incubation, higher cell concentration was used in short-term experiment in order to reach detection limit of plate counting (150 CFU/mL).

Whether D-tyrosine inhibits growth of *P. aeruginosa* was determined by comparing the total cell number in suspension and on membrane with or without addition of D-tyrosine in long-term experiment.

After incubation, the suspensions were sampled to determine the viable planktonic cell concentration by plate counting. Cells attached to the membrane were harvested by sonicating the membrane samples in 5 mL PBS buffer for 20 min in a sonicating bath followed by vortexing for 0.5 min

using a vortex mixer (Fisher Scientific, Pittsburg, PA). The cell suspension was then sampled and cell concentration measured by plate counting.

Table 4.2 Experimental matrix of attachment analysis.

	Time (h)	Cell Concentration (CFU/mL)	D-tyr Concentration (mM)	Number of Replicates
Short-term experiment	0.25	10^6	0	3
	0.25		30	3
	0.5		0	3
	0.5		30	3
	1		0	3
	1		30	3
Long-term Experiment	2	10^5	0	3
	2		3	3
	2		30	3
	4		0	3
	4		3	3
	4		30	3
	10		0	3
	10		3	3
	10		30	3
	24		0	3
	24		3	3
	24		30	3
	34		0	3
	34		3	3
	34		30	3

4.2.2 Bench scale dead-end filtration

The effectiveness of D-tyrosine in preventing biofilm formation in an actual filtration system was examined by bench scale dead-end filtration experiments. Unlike in the attachment experiments, the membrane feed water was under pressure in a membrane filtration system. Bacteria cells were forced to the membrane surface, which created a more favorable condition for cell adhesion to occur. The dead-end operation mode was chosen instead of cross-flow in order to accelerate cell attachment and biofilm development. Stirring was provided to generate hydraulic shear on the membrane surface in order to reduce concentration polarization.

Membrane filtration experiments were conducted in a bench-scale dead-end filtration system. The experimental setup (Figure 4.1) consists of a modified stainless steel stirred cell (400 mL in volume, Millipore, Bedford, MA), 3 stainless steel feed reservoirs (1 for pure water and 2 for fouling solution) and a four-way valve. In the system, water in reservoir was pressurized with a compressed nitrogen gas tank to pass through stirred cell. The four-way valve was used to control the nitrogen gas flow into a specific reservoir. The stirred filtration cell (Figure 4.2) was modified with a stainless steel cylinder replacing the original glass cylinder to allow high operation pressure. The membrane coupon was installed on the bottom of the filtration cell,

underneath which a nylon mesh was installed as the spacer. The effective surface area of membrane was 35.26 cm^2. A suspended magnetic stirrer was installed above the membrane to provide mixing. Two rubber o-rings were installed on the cap and the bottom respectively to seal the stirred cell.

The filtration system was first thoroughly cleaned with 2% SDS with pH adjusted to 10, and rinsed twice with ultrapure water. It was then sterilized with 200 proof ethanol followed by rinsing with sterilized ultrapure water to wash off remaining ethanol. One of the sterilized reservoirs was filled with 4 L ultrapure water, and the other two were filled with 4 L of electrolyte or synthetic wastewater, depending on the test condition. Membrane samples were sterilized with ethanol and rinsed 3 times with sterilized ultrapure water immediately before installation in the stirred cell.

The filtration experiments consisted of 4 steps: 1) Compaction. After sterilization, the membrane was compacted with sterilized ultrapure water for 2 hrs at 150 psi without stirring. 2) Conditioning. The membrane was conditioned by filtering the fouling solution for 2 hrs with stirring set at level 5. The permeate flux was adjusted to $2.20\pm0.02\times10^{-5}$m/s (100 ±10 psi) by modifying pressure. 3) Inoculation. When the permeate flux stabilized after 2 hours of conditioning, the pressure was released and the stirred cell was inoculated with 4 mL of *P. aeruginosa* liquid TSB culture (10^8 CFU/mL) to

obtain a cell concentration in the stirred cell of ~ 10^6 CFU/mL. This concentration was similar to the reported planktonic cell count in severely biofouled RO seawater treatment plant ($1.0\text{-}6.4\times10^6$ CFU/mL). (*35*) Then fouling solution was filtrated through membrane for 30 min without stirring to let the bacteria cells deposit on membrane surface. The filtration was stopped after 30 min by releasing the feed pressure. The remaining cell suspension in the stirred cell was carefully removed through outlet a (Figure 4.3) by pipetting, avoiding disturbing cells attached on the membrane surface. 4) Fouling. The fouling solution was filtered through the membrane for 20 hr at the same pressure as the conditioning step. Membrane permeate was collected in a 4 L plastic beaker on a digital balance (Denver Instrument, Arvada, CO)The accumulated weight of the membrane permeate was measured every 60 seconds by the digital balance, based on which membrane flux was calculated (equation 4.1),

$$J = \frac{\Delta M}{\rho \cdot A \cdot t} \quad \text{(Equation 4.1)}$$

where J is the permeate flux (m/s); ΔM is the mass increase of permeate during 60 s of time interval (g); ρ is the density of water (1×10^6 g/m^3); A is the effective membrane area (m^2); t is the time interval (60 s).

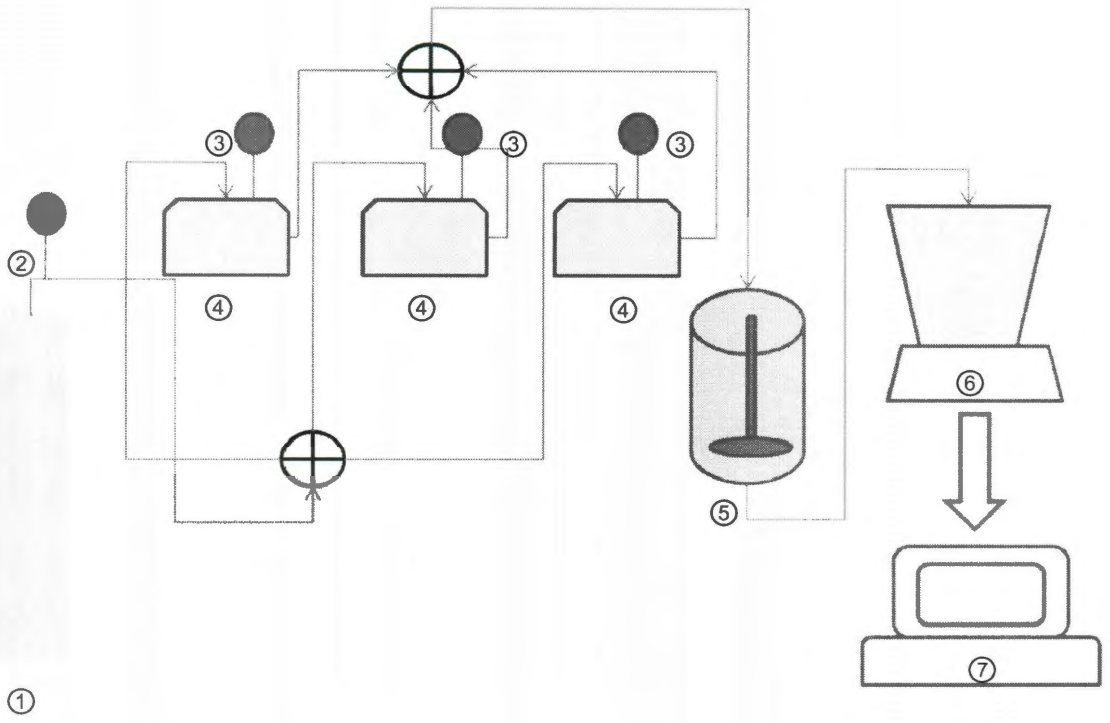

Figure 4.1 Schematic of bench scale dead-end filtration.

① Nitrogen gas; ② Regulator; ③ Pressure meter; ④ Stainless steel reservoirs for DI and fouling solution; ⑤ Stainless steel stirred cell with magnet stirrer, sealed with O rings; ⑥ Balance (0.01 g); ⑦ Computer.

After the filtration experiment, the fouled membrane was carefully taken out of the stirred cell and two 1 cm × 1 cm membrane samples were cut from it. The membrane specimens were gently rinsed twice with sterilized PBS buffer to remove unbounded cells. 100 μL 1 μg/mL DAPI solution was applied to the sample surface and kept for 10 min in dark. The excessive dye was removed with pipette carefully and the membrane specimen was rinsed again with sterilized PBS buffer. The membrane specimen was mounted on

a glass slide, covered with a cover slip and examined with an Olympus IX71inverted fluorescence microscope.

Two types of feed water with different nutrient levels were tested: an electrolyte solution supplemented with low concentration of nutrients and a synthetic wastewater with rich nutrient. The electrolyte solution has 0.1% addition of LB broth and 10 mM inorganic salts. Ca^{2+} was chosen since it was considered to increase bridging of alginate in EPS, leading to more severe fouling. Similar recipe of synthetic wastewater was used by Herzberg *et al.* in biofouling study of *P. aeruginosa* on polyamide composite membranes, where they observed significant biofouling and EPS production.(*16*) The HCO_3^- in the original recipe was removed to avoid possible inorganic scaling of $CaCO_3$ while additional NaCl was added to maintain the same ionic strength. The feed water compositions are shown in Table 4.3.

Table 4.3 Composition of feed water in dead-end filtration.

	NaCl (mM)	$CaCl_2$ (mM)	Trisodium Citrate (mM)	KH_2PO_4 (mM)	NH_4Cl (mM)	LB broth (V/V)	Ionic Strength (mM)	pH
Electrolyte	7	1				1/1000	10	6.70
Synthetic Wastewater	4.9	0.5	1.16	0.5	0.94	1/1000	14.8	6.83

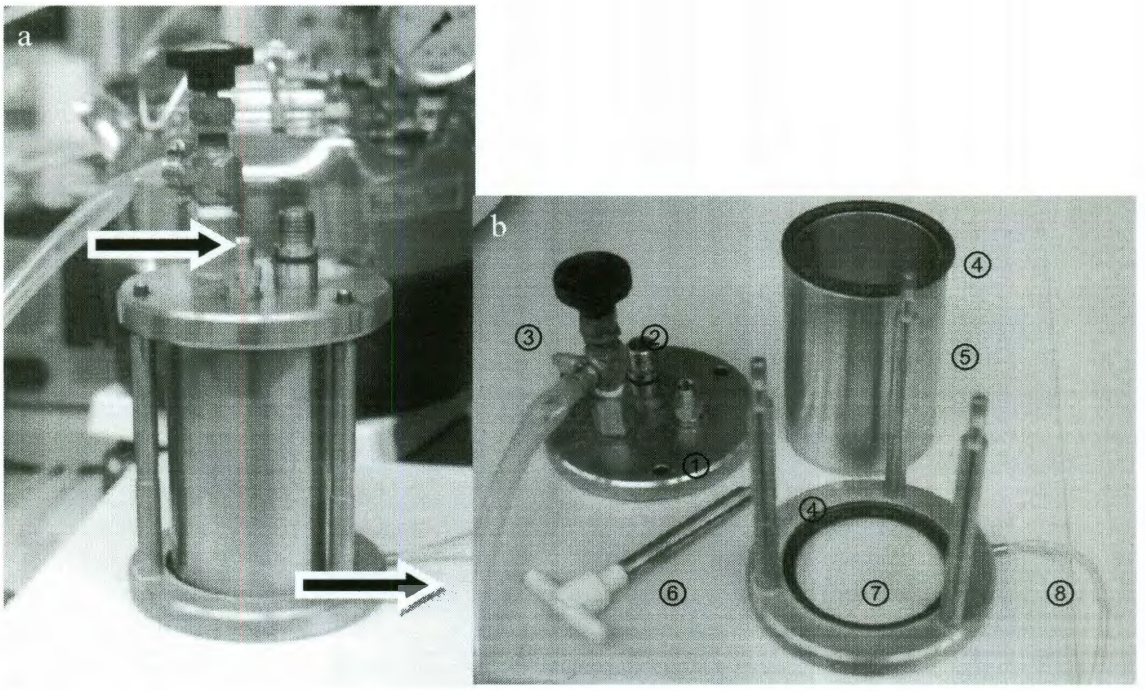

Figure 4.2 Image of stirred cell.

a) assembled stirred cell; arrows showing the direction of flow; b) parts of stirred cell, ① water inlet; ② water outlet a; ③gas releasing valve; ④rubber O-ring; ⑤ stainless steel cylinder; ⑥ magnet stirrer; ⑦ mesh; ⑧ water outlet b.

4.2.3Biofilm prevention screening of D-amino acids

To determine which D-amino acids have the ability to prevent biofilm formation of *P. aeruginosa*, a screening experiment was conducted.

A liquid TSB culture of *P. aeruginosa* was incubated at 37 °C for 24 hr to the late exponential phase. The suspension was diluted 10 times in LB broth to OD_{600} = 0.05 (10^7 CFU/mL, measured by plate counting using TSA

plates)(*89*). Aliquots of 100 μL bacteria suspension in LB broth supplemented with 0, 0.01, 0.1, 1 and 10 mM of a D-amino acid (three replicates for each condition) were added into the wells of a 96 well microtiter plate and the plate was incubated for 24 hrs at 37°C. The unbound cells were removed by rinsing with sterilized DI for 3 times. 25 μL of a 1% crystal violet solution was added to each well to stain the surface bound cells. The plate was then incubated for 45 min and the supernatant was discarded by pipetting after incubation. To remove the excessive dye, the wells were washed with sterilized DI for 3 times and the supernatant discarded. Then the crystal violet in the surface bound cells was extracted by incubating with 200 μL 95% ethanol for 45 min. 125 μL of the ethanol solution was then transferred from each well to a new 96-well polystyrene microtiter plate; The absorbance at 600 nm was measured with a Spectra Max Plus microtiter plate reader (Harlow Scientific, MA) to determine the concentration of the extracted crystal violet.

5 Result and discussion

5.1 Attachment analysis

5.1.1Effect of D-tyrosine on cell growth

The results of how D-tyrosine affects cell growth are shown in Figure 5.1. The total cell number was calculated by summing the planktonic and attached cell numbers and was plotted as a function of incubation time. The total cell number increased exponentially at the beginning and then slowed down, reaching the stationary phase after 24 hr of incubation. During the first 24 hr, D-tyrosine concentrations up to 30 μM had no impact on cell growth. After 34 hr incubation, although the total cell numbers in control and in the 3 μM were similar, the cell number in 30 μM appeared to be slightly higher. Since the total cell number in D-tyrosine supplemented wells was equal to or greater than in control wells, it could be concluded that D-tyrosine did not inhibit cell growth. The conclusion is in consistence with that of Kolodkin-Gal *et al.* (*6*) The addition of 30 μM D-tyrosine enhanced cell growth at 34 hr, possibly because D-tyrosine could be used as substrate for bacteria after other nutrient was depleted.

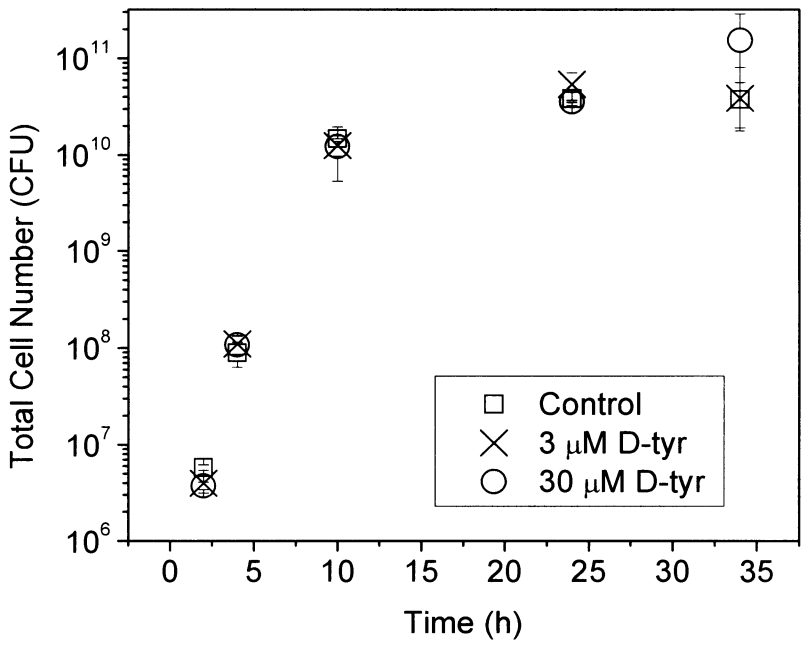

Figure 5.1 Effect of D-tyrosine on cell growth. Viable cell number was plotted with time. No obvious inhibition of cell growth was observed.

A short-term attachment analysis was conducted in LB media to assess the inhibitive effect of D-tyrosine on *P. aeruginosa* attachment. The initial reversible attachment of bacteria often happens in seconds and the irreversible attachment of bacteria happens in minutes. Cell attachment was measureed within one hour of incubation. The number of viable cells attached on membranes in different conditions was counted and compared in Figure 5.2. The ratio of the viable cell number on the 30 μM D-tyrosine treated membranes to that on the control membranes is shown in Figure 5.3.

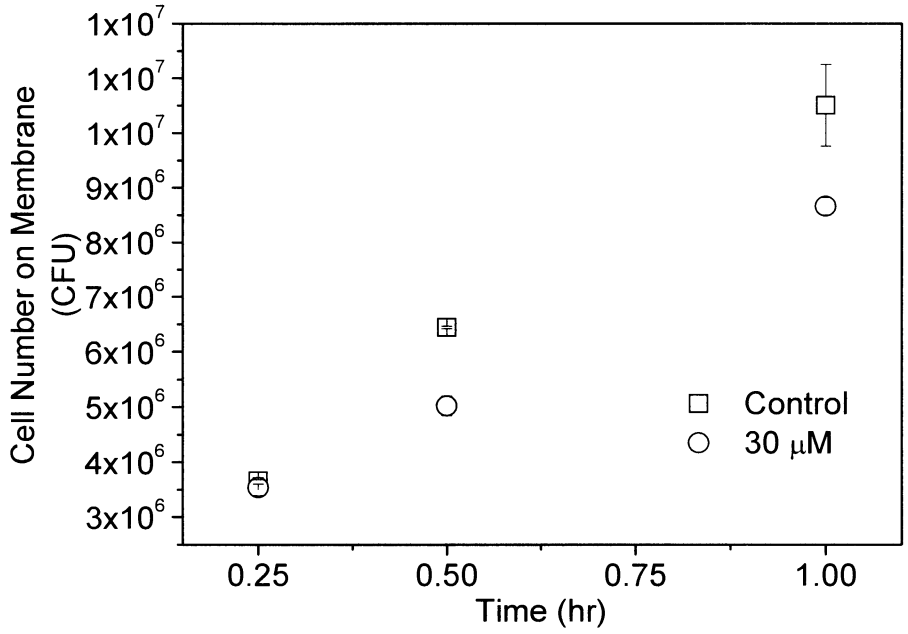

Figure 5.2 Viable cell numbers on NF 270 membrane surface with and without 30 μM D-tyrosine in the short-term cell attachment analysis (cell number on the y axis is on a log scale).

At 15 min, the attached cell number with and without D-tyrosine treatment was not statistically different, which indicates that D-tyrosine did not notably reduce the initial attachment of *P. aeruginosa* on NF270 membrane surface. This is possibly because 15 min is not enough for the bacteria to uptake the D-tyrosine in solution since the increase of viable cell number after 15 min was small. After 30 min, when the bacteria started to grow, samples treated with 30 μM D-tyrosine only had 78% viable cells on the membrane compared to the control group as shown in Figure 5.3. After 1

hour of incubation, the viable cell number on the 30 μM D-tyrosine treated membranes was only 82% of that on the control membranes.

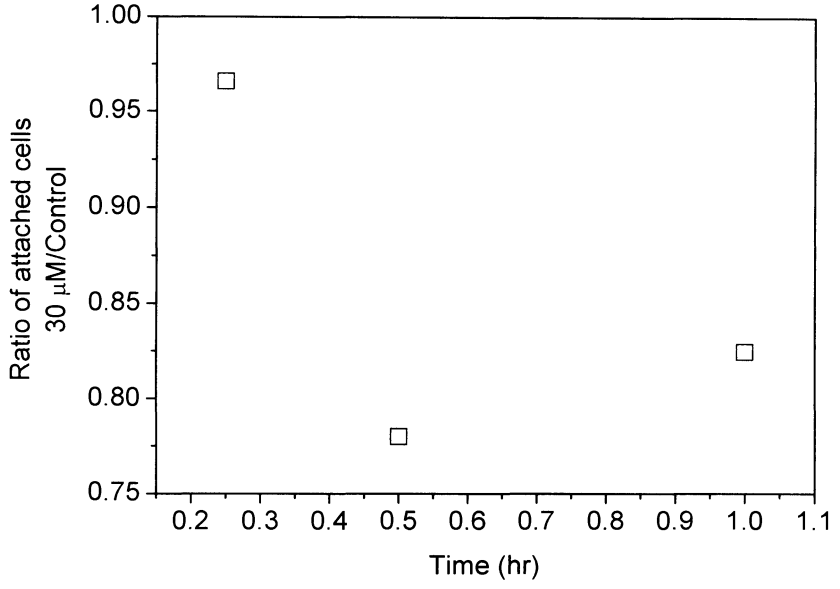

Figure 5.3 Ratio of viable cell number on 30 μM treated membrane to that of control (no D-tyrosine added).

Long-term experiments were also conducted to determine whether D-tyrosine could reduce cell growth on membrane surface (Figure 5.4). After incubated for 2 hrs, the numbers of attached cells on the 3 and 30 μM D-tyrosine treated membranes were half of that on the control membrane. However, after 4 hours of incubation, the cell numbers on control and treated membrane samples were not significantly different with a 90% confidential interval. The fact that reduction of cell attachment diminished when incubation time increased indicates that high cell population limited

the inhibitive effect of D-tyrosine. This suggests that the D-tyrosine concentration to cell number ratio plays an important role in the attachment inhibition mechanism. This is consistent with the hypothesis by Kolodkin-Gal *et al.* that D-tyrosine is taken up by *B. subtilis* and used in synthesis of peptidoglycan. This is also consistent with our hypothesis that D-tyrosine could substitute L-alanine in *P. aeruginosa* LPS. If the bacterium incorporates D-tyrosine into its cell components, each cell needs to have access to sufficient D-tyrosine in order to reduce attachment facilitated by LPS.

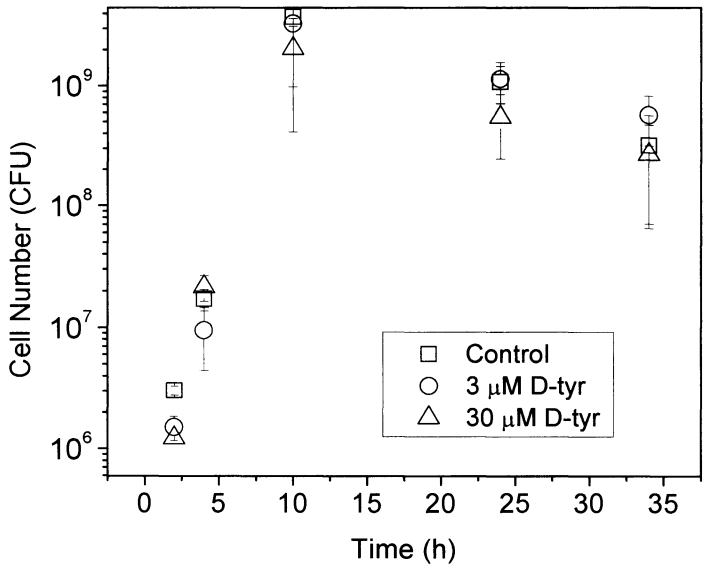

Figure 5.4 Long term attachment analysis of *P. aeruginosa* attachment on NF 270 membrane surface.

5.2 Bench scale dead-end filtration

Bench scale dead-end filtration experiments were performed to assess the effectiveness of D-tyrosine in inhibiting NF membrane biofouling during the filtration process. Figure 5.5 presents a typical data set collected during one experiments, showing the flux behavior during different stages of the experiment. During compaction at 150 psi, the clean water flux was 3.8×10^{-5} m/s. When filtering the fouling solution in the conditioning stage (100 psi), the flux was reduced to about half of clean water flux due to concentration polarization. Further flux decline after inoculation of the system was attributed to growth of the bacterial cells on the membrane surface.

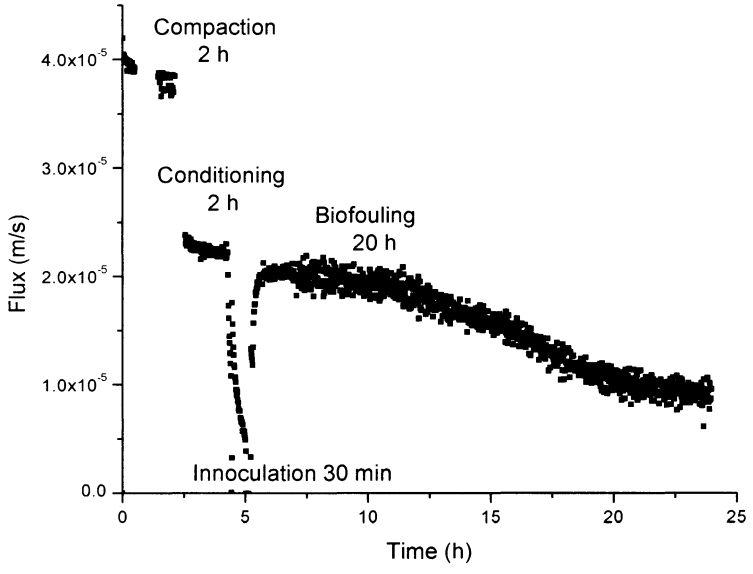

Figure 5.5 Permeate flux change during one filtration experiment (The feed water is the low nutrient electrolyte without D-tyrosine. Applied pressure was 150, 100 and 100 psi for the compacting, conditioning and fouling stages, respectively)

5.2.1 Low nutrient feed water

The permeate flux change during filtration of the low nutrient fouling solution supplemented with different concentrations of D-tyrosine is shown in Figure 5.6. Each data point on the flux curve is the average of 10 flux data points recorded. To compare flux decline in different conditions, the flux during the fouling stage was normalized by the stable flux in the conditioning stage ($2.20 \pm 0.02 \times 10^{-5}$m/s). With no D-tyrosine in the feed water, the flux declined by 64% after 20 hr of filtration. After about 6 hr of a lag phase when flux decline was not obvious, the permeate flux decreased rapidly in the next 9 hr. The rate of flux decline slowed down after 15 hr of filtration and the permeate flux gradually dropped to 8.64×10^{-6} m/s at about 20 hr. There was a lag phase of flux decline in the first 6 hrs because the bacteria need time to reproduce and to form a thick enough layer on membrane surface before notable flux decline due to the increased hydraulic resistance and biofilm enhanced concentration polarization can be observed. This also suggests that measurement of permeate flux cannot detect biofouling in the early stage. After 15 hr, the biofilm development was limited by low nutrient level and thus the flux reaches quasi steady state gradually. Addition of D-tyrosine in the feed solution significantly reduced membrane flux decline, and this effect increased with increasing D-tyrosine

concentration. With continuous feed of 30 μM D-tyrosine, flux decline was completely prevented. This indicates that the biofouling control efficacy of D-tyrosine depends on D-tyrosine to cell concentration ratio. Such dependence on D-tyrosine concentration supports the hypothesis that D-amino acids are taken up by bacterial cells. There may exist a minimum D-amino acid to cell concentration ratio, below which effective biofilm control cannot be achieved.

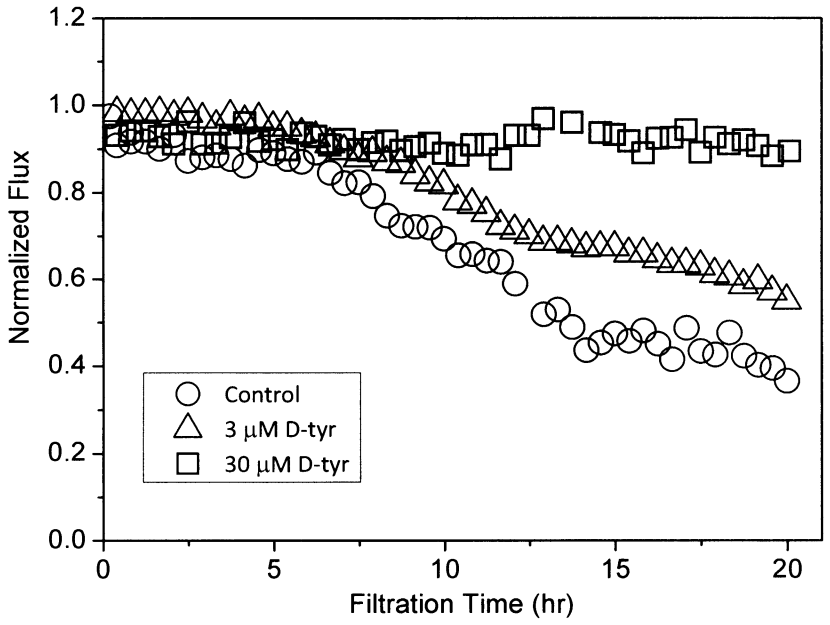

Figure 5.6 Normalized flux during filtration of the electrolyte fouling solution supplemented with different concentrations of D-tyrosine.

The resistance of membrane was calculated by Darcy's law (Equation 5.1) coupled with resistance in series model (Equation 5.2):

$$R = \frac{dp}{\mu J} \text{ (Equation 5.1)}$$

where R is membrane resistance (m^{-1}); dp is trans membrane pressure change (Pa); μ is viscosity of water at 20 °C (1.005×10^{-3} Pa·S); J is membrane permeate flux.

$$\text{During compaction, } R = R_m \quad \text{(Equation 5.2)}$$

$$\text{During conditioning, } R = R_m + R_{cp} \quad \text{(Equation 5.3)}$$

$$\text{During fouling, } R = R_m + R_f \quad \text{(Equation 5.4)}$$

In Equation 5.2 to 5.4, R is total membrane resistance; R_m is membrane intrinsic resistance; R_{cp} is resistance caused by concentration polarization; and R_f is the resistance caused by biofouling. R_m was calculated from clean water flux during compaction where R_f was zero. The resistance caused by fouling was calculated as the total resistance after 20 hrs fouling excluding membrane intrinsic resistance. R_{cp} was calculated by resistance at conditioning stage excluding R_m. Biofouling increases hydraulic resistance of membrane and enhances the concentration polarization on membrane surface, where both contribute to flux decline.(*16*) So the resistance caused by concentration polarization cannot be isolated from fouling resistance.

The anti-biofouling effect of D-tyrosine treatment can be seen in Figure 5.7, which compares the different contributors to the overall hydraulic resistance

of the membrane. The intrinsic membrane resistance and concentration polarization caused membrane resistance were similar in each condition. However, the addition of D-tyrosine reduced fouling resistance significantly.

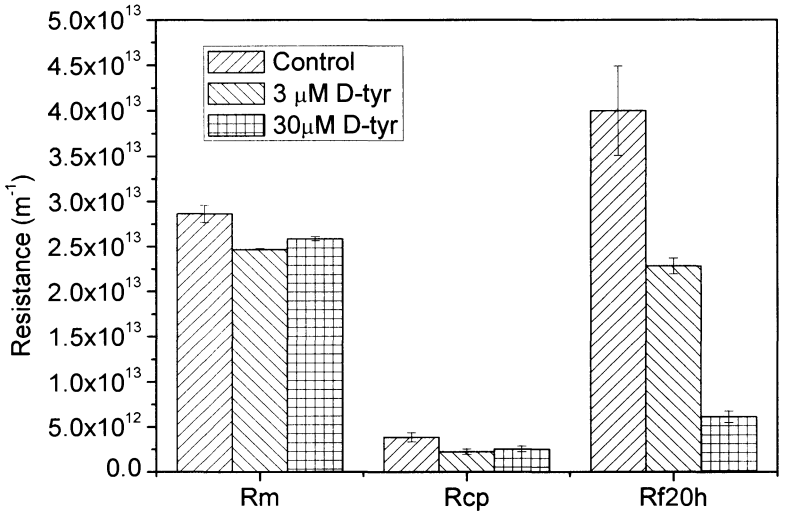

Figure 5.7 Comparison of different contributors to the overall membrane resistance (m^{-1}). R_m: clean membrane resistance; R_{cp}: resistance due to concentration polarization; R_{f20h}: total resistance due to fouling (including the hydraulic resistance of the fouling layer and the resistance due to concentration polarization) after 20 hr filtration of the fouling solution.

Fluorescence microscope images of the fouled membranes were taken to view the cell attachment on the membrane surface. As is shown in Figure 5.8 (a), very high cell density was found on the membrane surface after the control experiment, and multi-layer coverage was observed in various locations on the surface. The membranes treated with D-tyrosine (Figures 5.8 b to d), on the other hand, had very few cells attached, and the number of cells attached decreased with increasing D-tyrosine concentration. No microcolonies or cell aggregates were found on these membranes. Since D-

tyrosine does not inhibit *P. aeruginosa* growth (Figure 5.1), this suggests cell-cell and cell-membrane adhesion in the presence of D-tyrosine 30μM and 3μM experiments. These results are consistent with the membrane flux data, which show greatly decreased flux decline when D-tyrosine was added to the feed water, suggesting that reduced cell-membrane and cell-cell adhesion is the reason for the lower fouling rate.

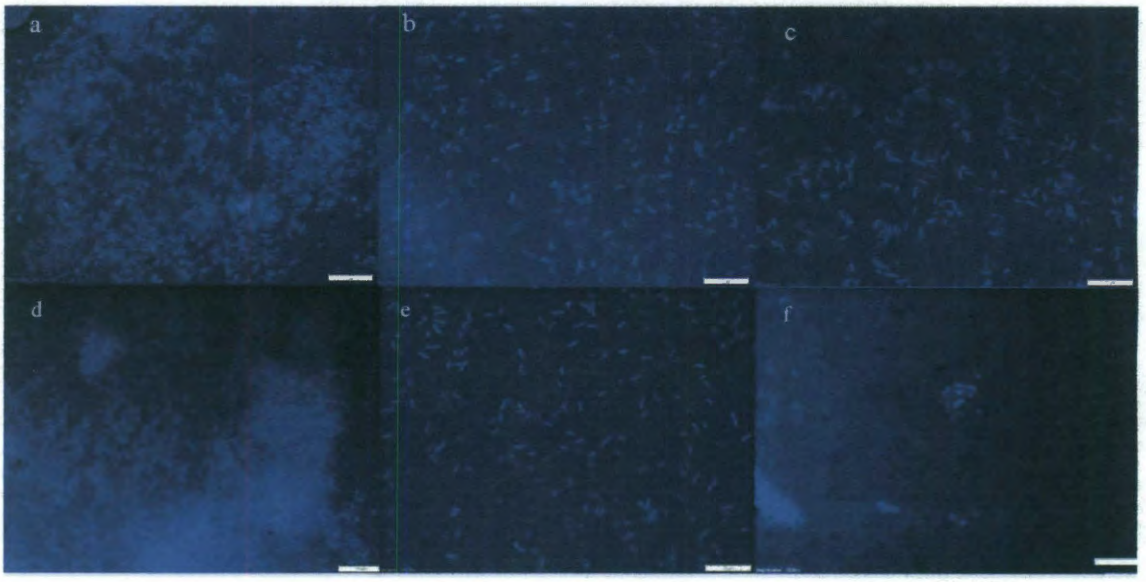

Figure 5.8 Fluorescence images of fouled membrane after filtration of low nutrient feed water: a) control; b) 3 μM D-tyr; c) 30 μM D-tyr, and high nutrient feed water: d) control; e) 3 μM D-tyr; f) 30 μM D-tyr. Scale bars are 10 μm.

5.2.2 High nutrient feed water

To simulate the biofouling condition in actual wastewater treatment by nanofiltration, synthetic wastewater containing high concentrations of nutrients was used as the fouling solution.

Comparison of fouling flux in synthetic wastewater supplemented with different concentrations of D-tyrosine is shown in Figure 5.9. The permeate flux is normalized by that during conditioning ($2.20 \pm 0.02 \times 10^{-5}$m/s). Without D-tyrosine, the permeate flux declined by 70% in 20 hr. Addition of 3 or 30 μM D-tyrosine in the feed water only slightly reduced membrane flux decline, and the concentration of D-tyrosine had not notable effect on the flux behavior.

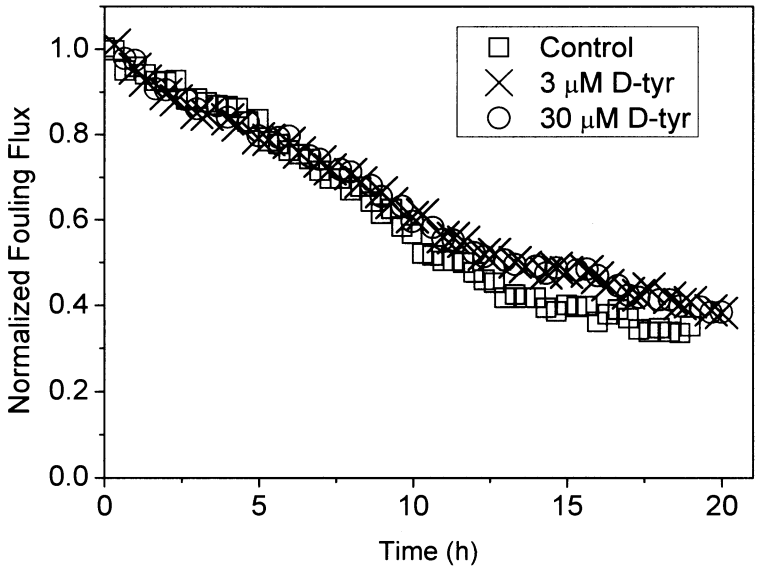

Figure 5.9 Comparison of normalized permeate flux during filtration of the synthetic wastewater with and without D-tyrosine.

Interestingly, fluorescent microscope imaging revealed drastic difference in cell attachment between the fouled membranes. Figure 5.8 shows the fluorescent microscope images of the membrane surfaces after filtration with and without D-tyrosine. Very high density of *P. aeruginosa* cells was found

on the control sample (Figure 5.8c). Although the surface was not completely covered by a biofilm, there were muti-layer microcolonies and large clusters of cells. The cell density was much greater than that observed after filtration of the low nutrient feed water because of higher cell growth rate at the higher nutrient level. In contrast, few cells were found on the membrane surfaces after filtration in the presence of D-tyrosine. Even at a concentration as low as 3 μM D-tyrosine, *P. aeruginosa* attachment on the membrane surface was greatly reduced (Figure 5.8d). Fewer cells were observed on 30 μM membrane. These results clearly show that continuous feed of D-tyrosine inhibits *P. aeruginosa* cell attachment on NF 270 membrane surface in dead-end filtration operation conditions even at high cell concentrations due to the high nutrient level. However, flux decline was not prevented by D-tyrosine, as shown in Figure 5.9. This is most likely due to the high cell concentration in the suspension. With the high nutrient level in the synthetic wastewater, the viable planktonic cell concentration in the stirred cell reached 10^8 CFU/mL (measured after fouling with plate counting) in all 3 filtration experiments. In dead-end operation mode, the bacteria cells were brought to the membrane surface by the convective permeate flow and held against the membrane surface by the permeate drag. The high concentration of bacteria in the suspension resulted in fast accumulation of

bacterial cells on membrane surface. Even if the cells are not irreversibly attached to the membrane surface or to each other, the cell layer accumulated on the membrane surface could lead to flux decline due to cake enhanced concentration polarization and the hydraulic resistance of the cell layer itself.(*16*) In this case, the bacterial cells behave similarly to colloidal foulants; the cells in the "cake" layer are re-dispersed upon release of the pressure when filtration is stopped. Herzberg *et al.* reported approximately 50% permeate flux decline using a synthetic wastewater similar to that used in this study containing dead *P. aeruginosa* cells (10^9 cell/mL) on a commercial RO membrane (LFC-1) after 27.8 hr filtration (1.6 L permeate was collected), even though dead cells obviously were not able to form biofilms; the flux decline was mainly attributed to the dead cell cake enhanced concentration polarization due to hindered back diffusion of salt ions.(*16*, *25*) Therefore even though the cell adhesion was reduced by D-tyrosine, severe flux decline was still observed. Since the deposited cells treated with D-tyrosine were not irreversibly attached to the membrane surface, when the pressure was released, the deposited cell layer was easily removed from membrane surface. These results suggest that D-tyrosine could alleviate biofouling by rendering the biofouling layer reversible and hence easily removed during membrane cleaning.

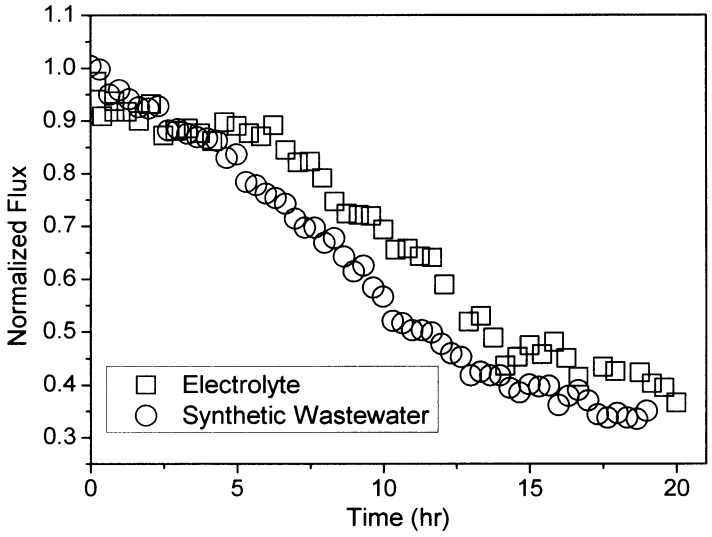

Figure 5.10 Fouling flux in high and low nutrient feed water without D-tyrosine added.

In Figure 5.10, flux declines in fouling stage of the high nutrient solution and the low nutrient solution without D-tyrosine was compared. Since the nutrient level in synthetic wastewater is much higher than in electrolyte, the bacteria growth is more active hence more biomass is expected to accumulate on membrane surface within same fouling time. Compared to the filtration experiments performed using the electrolyte solution, significantly more flux decline during the first 6 hrs occurred. There was no lag phase in flux decline as that observed during filtration of the low nutrient feed water. In synthetic wastewater, the initial flux decline rate was much greater than in the electrolyte solution. This is due to the greater growth rate and hence higher cell concentration in the feed water with elevated nutrient concentrations. However, the quasi-steady state flux after fouling was

similar in both conditions, therefore the flux decline caused by biofouling in 20 hr in both conditions were similar.

5.3 Screening of D-amino acids for biofilm inhibition potential

α-amino acids are most commonly found in nature. There are 20 α-amino acids that are used for protein synthesis. D-isomers of 19 α-amino acids were tested for their biofilm inhibition efficiency (Glycine does not have optical isomers). The concentrations of D-amino acids tested ranged from 10 μM to 10 mM except for D-tyrosine, which had low solubility in water (0.453 g/L). Therefore, the concentration range of D-tyrosine tested is 1 to 100 μM.

As described in Chapter 4, the absorbance at 600 nm of the ethanol extracted crystal violet solution was used as an indirect measure for attached biomass. The results were normalized by that of the control, to which no D-amino acids were added. A normalized absorbance lower than 1 indicates inhibition of biofilm formation by the D-amino acid added. All 19 D-amino acids inhibited biofilm formation at high concentrations of 10 mM and 1 mM (Figure 5.11and Figure 5.12). The comparisons of biofilm inhibition effect of different D-amino acids at 10 mM, 1 mM, 100 μM and 10 μM are shown

in Figure 5.11 to Figure 5.14 respectively. At 10 mM (shown in Figure 5.11), D-asparagine and D-glutamic acid showed the best performances in reducing *P. aeruginosa* biofilm on polystyrene surface, where attached biomass was only 20.3% and 24.5% of that of the control. D-threonine, on the other hand, had the least effect on biofilm formation. The biomass attached on the polystyrene surface was 87.5% of that in the control samples. At 1 mM, all D-amino acids showed biofilm inhibition ability except for D-threonine and D-isoleucine. In the presence of these two D-amino acids, the attached biomass was more than that in the controls. Such over growth phenomenon was also observed at 100 μM and 10 μM concentrations. At 100 μM, D-histidine and D-isoleucine induced more attached biomass than the controls while at 10 μM, D-threonine, D-aspartic acid, D-histidine and D-asparagine resulted in more attached biomass than the controls. The possible reason for the enhanced biomass attachment is that D-amino acids are utilized by the bacteria as substrate. In figure 5.15, the biofilm inhibition effect for each D-amino acid at different concentrations was compared, which generally increase with the increase of D-amino acid concentration. Some D-amino acids showed higher efficiency than D-tyrosine at low concentrations, for instance, D-leucine. D-leucine is more soluble in water which indicated that higher dosage is possible to improve biofilm inhibition performance.

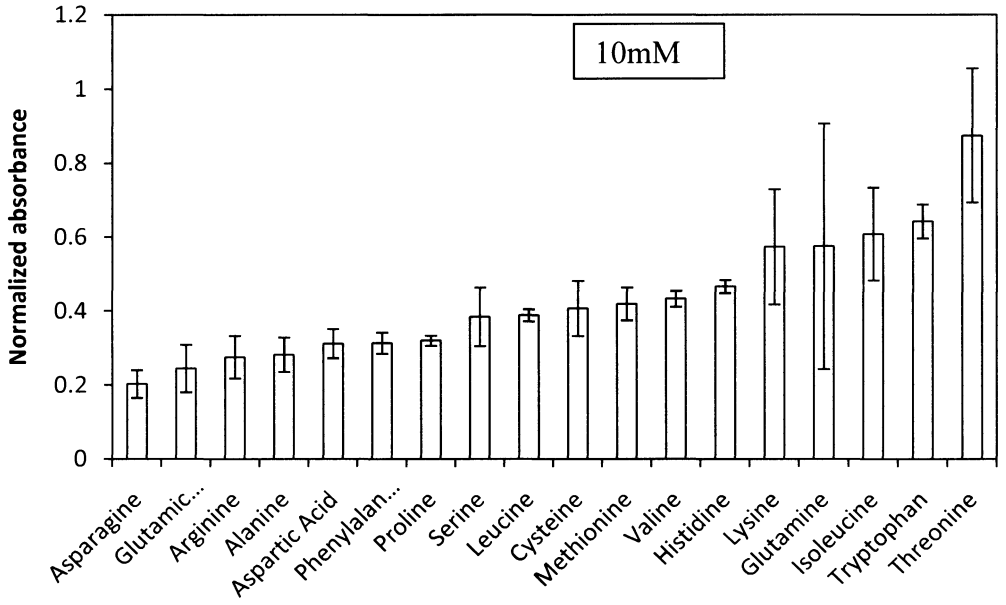

Figure 5.11 Normalized biomass attachment in the presence of 18 D-amino acids at 10 mM.

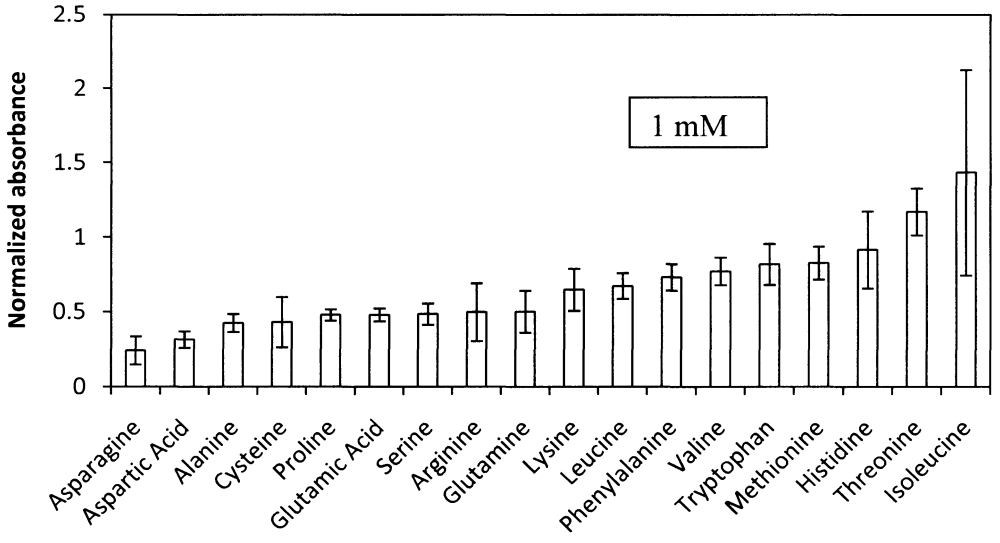

Figure 5.12 Normalized biomass attachment in the presence of 18 D-amino acids at 1 mM.

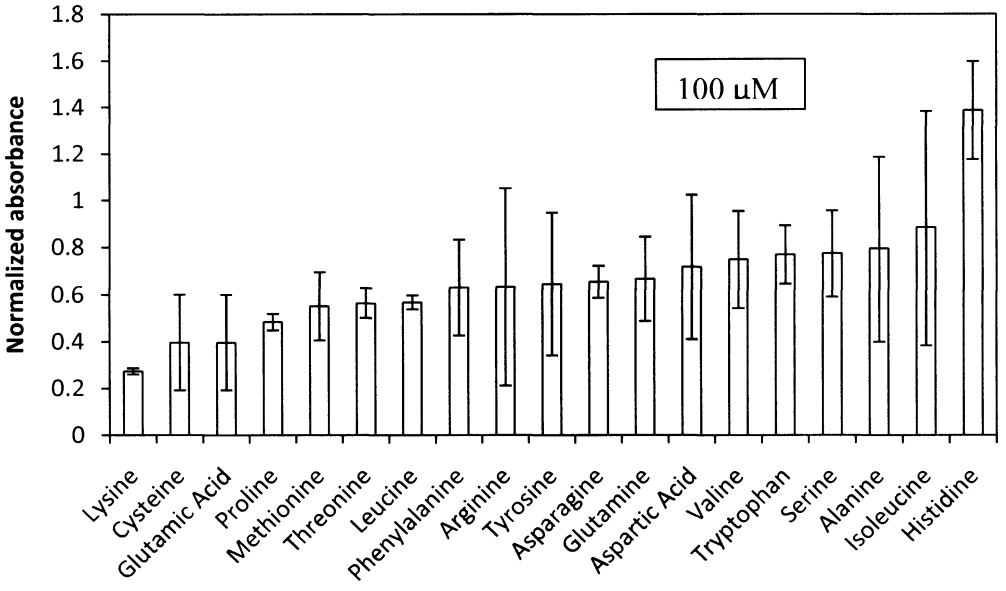

Figure 5.13 Normalized biomass attachment in the presence of 18 D-amino acids at 100μM.

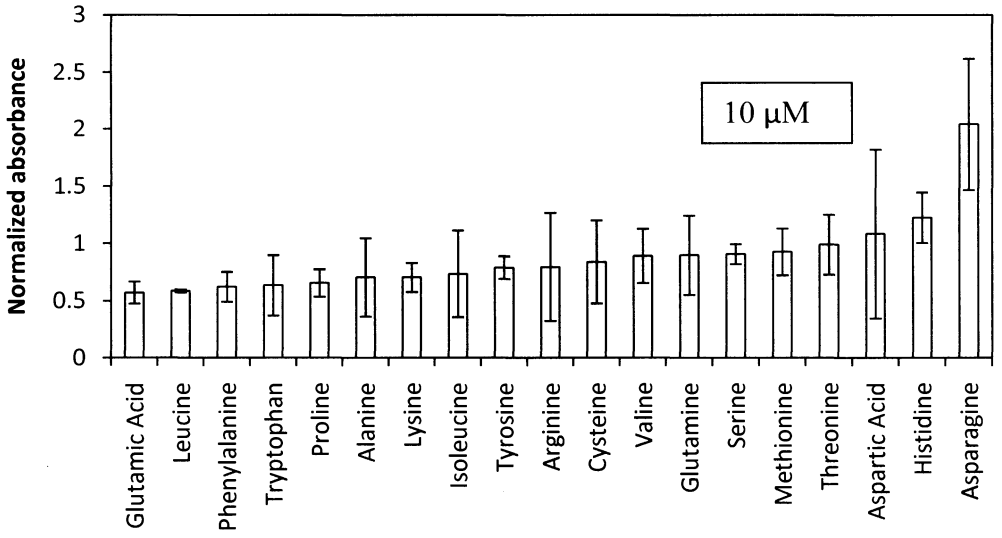

Figure 5.14 Normalized biomass attachment in the presence of 18 D-amino acids at 10 μM.

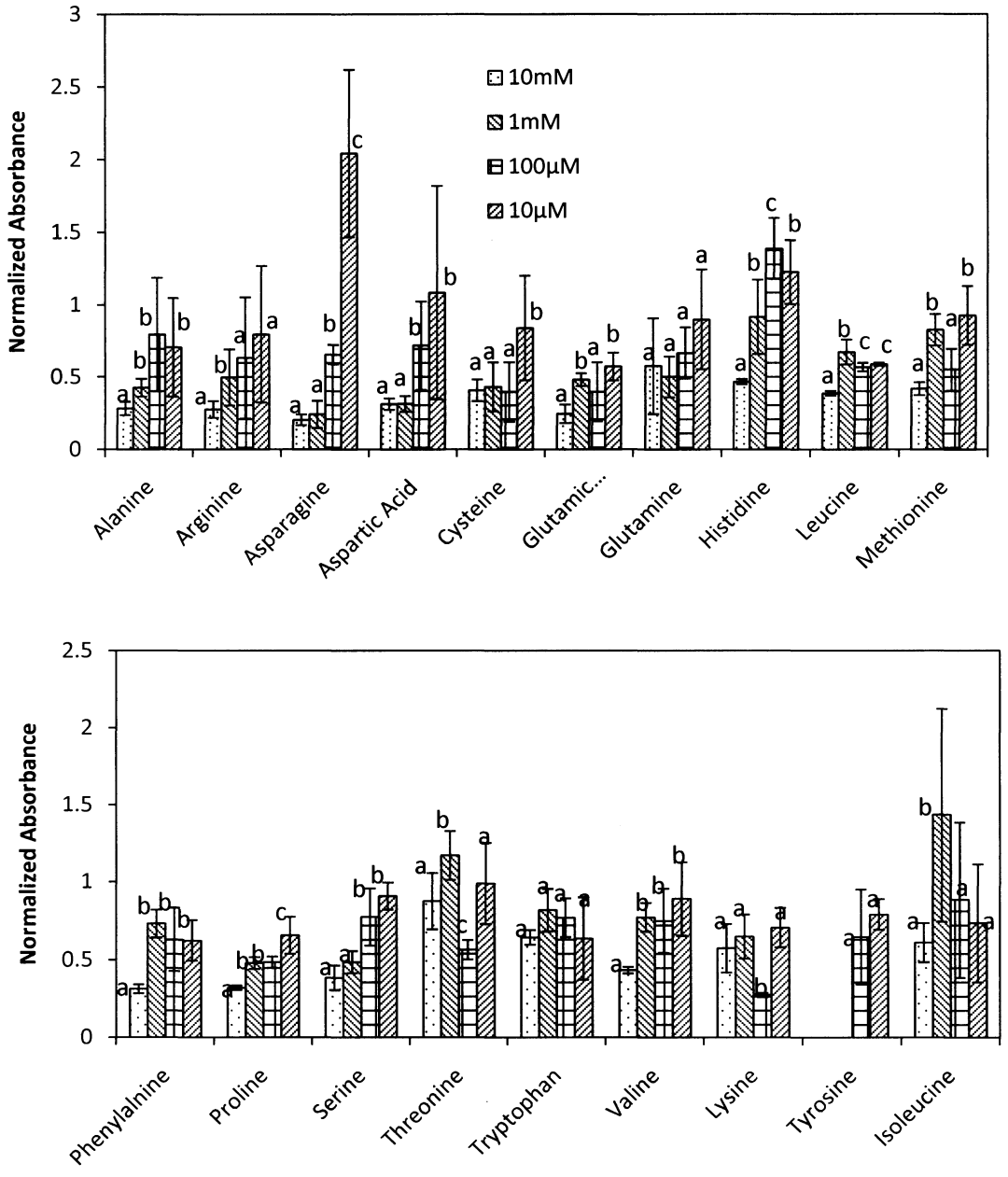

Figure 5.15 Biofilm inhibition screening of 19 D-amino acids.The statistical difference between different concentrations was determined by Student't test (t_{90}=1.83). Data points bearing the same text labels are not statistically different.

5.4 Hypothesized mechanisms

Results from this study indicate that D-amino acids to cell concentration ratio affects biofouling inhibition efficiency, which suggest that D-amino acids need to be taken up by or associated with bacteria to reduce bacterial attachment and hence biofouling. It is suspected that the D-amino acids can be incorporated into LPS of *P. aeruginosa* cell wall in substitution of L-alanine. In *P. aeruginosa,* the core polysaccharide that anchors LPS on the outer membrane contains L-alanine. It is possible that in a *P. aeruginosa* biofilm, D-amino acids work in similar manner as with *B. subtilis*: D-amino acids replace L-alanine in LPS, leading to loss of LPS from the outer membrane. Loss of LPS could disable the cell-cell and cell-membrane attachment in *P. aeruginosa*.

The lack of dependency of biofilm inhibition effect on the D-amino acid to cell concentration ratio for D-leucine, D-tryptophan, D-lysine and D-isoleucine suggests that there might be other mechanisms through which D-amino acids inhibit biofilm formation. For example, D-amino acids have been hypothesized to work as signal molecules for bacteria.(*5*) Once the concentration of the signal molecule reaches the effective level, further increase in the concentration does not have much impact.

6 Conclusions and Future Research

This study finds that all 19 natural D-amino acids are able to inhibit *P. aeruginosa* biofilm formation. D-tyrosine inhibits *P. aeruginosa* attachment to polyamide NF membrane surfaces in LB media at concentrations as low as 3 μM. Continuous addition of D-tyrosine to membrane feed water is effective in controlling biofouling in NF systems by reducing cell-membrane and cell-cell attachment.

The inhibitive effect of most D-amino acids on *P. aeruginosa* biofilm formation depends on the D-amino acid to cell concentration ratio. It is hypothesized that D-amino acids are taken up by *P. aeruginosa*. Comparison with previous studies (*5*, *6*) reveals that different types of bacteria may respond differently towards the same D-amino acid.

Further research is needed to determine the mechanisms through which D-amino acids inhibits bacterial biofilms, and to developed practical ways to apply D-amino acids for biofouling control in membrane systems.

It is important to determine the mechanisms of biofilm inhibition by D-amino acids for two reasons. It could enable us to understand more about the function of D-amino acids in bacteria cell wall, the current knowledge of which is still inadequate. In addition, understanding the mechanisms will

allow us to determine which D-amino acid or D-amino acid mixtures are more efficient and widely effective in bacterial biofilm prevention, which may lead to novel strategies of biofouling control. To test the hypothesis that D-amino acids are incorporated into *P. aeruginosa* cell wall, ^{14}C labeled D–amino acids can be used and concentration and position can be measured by radioactivity. To test the hypothesis that D-amino acids substitute L-alanine in core polysaccharide in LPS of *P. aeruginosa*, excessive amount of L-alanine could be added to *P. aeruginosa* culture together with the D-amino acid to find out if the inhibitive effect is reduced or eliminated.

A lot more work needs to be conducted before D-amino acids can be applied for biofouling control in membrane filtration systems. First, the effectiveness of biofilm prevention of D-amino acids towards bacteria species other than *P. aeruginosa* that are known to cause biofouling in membrane systems should be determined. Although widely used as model bacteria for membrane biofouling, *P. aeruginosa* was considered not the dominant bacteria on seawater desalination reverse osmosis (SWRO) membranes according to recent studies.(*90*, *91*) Instead, *Leucothrix mucor* and *Ruegeria* species were reported to be the dominant bacteria using isolation independent method by Zhang *et al.*(*91*) Second, the optimal selection and concentration of D-amino acids in biofilm prevention should be determined. A mixture of D-amino

acids has been shown to be much more effective than its individual components.(*6*) The crystal violet assay can be used as a fast screening method to identify optimal mixtures. Third, whether D-amino acids could inhibit multi-species biofilm containing a wide spectrum of bacteria should be tested. Bacteria that have been reported to be susceptible to D-amino acids treatment include *P. aeruginosa*, *B. subtilis* and *S. aureus*.(*7*) These bacterial species were tested in pure cultures. Whether D-amino acids have similar effects on other bacterial pure cultures or mixed cultures is unknown. Fourth, the filtration experiments were only conducted with NF 270 membrane, which is fairly smooth and has low fouling potential. Since D-amino acids inhibit biofouling by reducing cell-membrane adhesion, the surface properties of the membrane may play a role. More NF and RO membranes with different surface properties need to be tested. Finally, different approaches of applying D-amino acids in biofouling control should be evaluated, such as direct addition into feed water, incorporation into the membrane and use in chemical cleaning. Continuous addition to the feed water is not desired because it would require a large amount of D-amino acids, which is very expensive.

7 Reference

1. FILMTEC Reverse Osmosis Membranes Technical Manual.

2. Yeon, K.-M., Cheong, W.-S., Oh, H.-S., Lee, W.-N., Hwang, B.-K., Lee, C.-H., Beyenal, H., and Lewandowski, Z. (2009) Quorum sensing: a new biofouling control paradigm in a membrane bioreactor for advanced wastewater treatment., *Environmental science & technology 43*, 380-5.

3. Xiong, Y., and Liu, Y. (2010) Biological control of microbial attachment: a promising alternative for mitigating membrane biofouling., *Applied microbiology and biotechnology 86*, 825-37.

4. Kim, J.-H., Choi, D.-C., Yeon, K.-M., Kim, S.-R., and Lee, C.-H. (2011) Enzyme-Immobilized Nanofiltration Membrane To Mitigate Biofouling Based on Quorum Quenching., *Environmental science & technology* 1601-1607.

5. Lam, H., Oh, D.-C., Cava, F., Takacs, C. N., Clardy, J., Pedro, M. a de, and Waldor, M. K. (2009) D-amino acids govern stationary phase cell wall remodeling in bacteria., *Science (New York, N.Y.) 325*, 1552-5.

6. Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010) D-Amino Acids Trigger Biofilm Disassembly, *Science 328*, 627-629.

7. Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010) D-Amino Acids Trigger Biofilm Disassembly-Supporting Material, *Science 328*, 627-629.

8. Shon, H. K., Vigneswaran, S., and Snyder, S. a. (2006) Effluent Organic Matter (EfOM) in Wastewater: Constituents, Effects, and Treatment, *Critical Reviews in Environmental Science and Technology 36*, 327-374.

9. Kim, A. S., Contreras, A. E., Li, Q., and Yuan, R. (2009) Fundamental mechanisms of three-component combined fouling with experimental verification., *Langmuir□: the ACS journal of surfaces and colloids 25*, 7815-27.

10. Ridgway, H., Ishida, K., Rodriguez, G., Safarik, J., Knoell, T., and Bold, R. (1999) Biofouling of membranes: membrane preparation, characterization, and analysis of bacterial adhesion., *Methods in enzymology 310*, 463-94.

11. Flemming, H., Schaule, G., Griebe, T., Schmitt, J., and Tamachkiarowa, A. (1997) Biofouling—the Achilles heel of membrane processes, *Desalination 113*, 215-225.

12. Speth, T. F., Summers, R. S., and Gusses, A. M. (1998) Nanofiltration Foulants from a Treated Surface Water, *Environmental Science & Technology 32*, 3612-3617.

13. Mansouri, J., Harrisson, S., and Chen, V. (2010) Strategies for controlling biofouling in membrane filtration systems: challenges and opportunities, *Journal of Materials Chemistry 20*, 4567.

14. Kang, S., Subramani, a, Hoek, E., Deshusses, M., and Matsumoto, M. (2004) Direct observation of biofouling in cross-flow microfiltration: mechanisms of deposition and release, *Journal of Membrane Science 244*, 151-165.

15. Donlan, R. M. (2002) Biofilms: microbial life on surfaces., *Emerging infectious diseases 8*, 881-90.

16. Herzberg, M., and Elimelech, M. (2007) Biofouling of reverse osmosis membranes: Role of biofilm-enhanced osmotic pressure, *Journal of Membrane Science 295*, 11-20.

17. O'Toole, G., and Kolter, R. (1998) Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development, *Molecular Microbiology 30*, 295-304.

18. Kolter, Geoge A. O'Toole, R. (1998) Flagellar and twitching motility are necessary for Peudomonous aeruginosa biofilm development, *Molecular microbiology 30*, 295-304.

19. Lind, M. L., Ghosh, A. K., Jawor, A., Huang, X., Hou, W., Yang, Y., and Hoek, E. M. V. (2009) Influence of zeolite crystal size on zeolite-polyamide thin film nanocomposite membranes., *Langmuir□: the ACS journal of surfaces and colloids 25*, 10139-45.

20. Loosdrecht, M. C. van, Lyklema, J., Norde, W., and Zehnder, a J. (1990) Influence of interfaces on microbial activity., *Microbiological reviews 54*, 75-87.

21. Liao, B. Q., Allen, D. G., Droppo, I. G., Leppard, G. G., and Liss, S. N. (2001) Surface properties of sludge and their role in bioflocculation and settleability., *Water research 35*, 339-50.

22. Liao, B. Q., Bagley, D. M., Kraemer, H. E., Leppard, G. G., and Liss, S. N. (2004) A review of biofouling and its control in membrane separation bioreactors., *Water environment research□: a research publication of the Water Environment Federation 76*, 425-36.

23. Karatan, E., and Watnick, P. (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms., *Microbiology and molecular biology reviews□: MMBR 73*, 310-47.

24. Subramani, A., and Hoek, E. M. V. (2010) Biofilm formation, cleaning, re-formation on polyamide composite membranes, *Desalination*, Elsevier B.V. *257*, 73-79.

25. Herzberg, M., Kang, S., and Elimelech, M. (2009, June 15) Role of extracellular polymeric substances (EPS) in biofouling of reverse osmosis membranes., *Environmental science & technology*.

26. Hoek, E. M. V., and Elimelech, M. (2003) Cake-enhanced concentration polarization: a new fouling mechanism for salt-rejecting membranes., *Environmental science & technology 37*, 5581-8.

27. Hijnen, W. a M., Biraud, D., Cornelissen, E. R., and Kooij, D. van der. (2011) Threshold Concentration of Easily Assimilable Organic Carbon in Feedwater for Biofouling of Spiral-Wound Membranes., *Environmental science & technology 43*, 4890-4895.

28. Baker, J., and Dudley, L. (1998) Biofouling in membrane systems — A review1, *Desalination 118*, 81-89.

29. Ivnitsky, H., Katz, I., Minz, D., Shimoni, E., Chen, Y., Tarchitzky, J., Semiat, R., and Dosoretz, C. (2005) Characterization of membrane biofouling in nanofiltration processes of wastewater treatment, *Desalination 185*, 255-268.

30. Hong, S., and Elimelech, M. (1997) Chemical and physical aspects of natural organic matter (NOM) fouling of nanofiltration membranes, *Journal of Membrane Science 132*, 159-181.

31. Abu Seman, M. N., Khayet, M., and Hilal, N. (2011) Development of antifouling properties and performance of nanofiltration membranes modified by interfacial polymerisation, *Desalination*, Elsevier B.V. *273*, 36-47.

32. Alahmad, M., Abdulaleem, F., Mutiri, A., and Ubaisy, A. (2000) Biofouling in RO membrane systems Part 1: Fundamentals and control1, *Desalination 132*, 173-179.

33. Chua, K., Hawlader, M., and Malek, a. (2003) Pretreatment of seawater: Results of pilot trials in Singapore, *Desalination 159*, 225-243.

34. Griebe, T., and Flemming, H. (1998) Biocide-free antifouling strategy to protect RO membranes from biofouling1, *Desalination 118*, 153-156.

35. Schneider, R., Ferreira, L., Binder, P., Bejarano, E., Goes, K., Slongo, E., Machado, C., and Rosa, G. (2005) Dynamics of organic carbon and of bacterial populations in a conventional pretreatment train of a reverse osmosis unit experiencing severe biofouling, *Journal of Membrane Science 266*, 18-29.

36. Hu, J. Y., Song, L. F., Ong, S. L., Phua, E. T., and Ng, W. J. (2005) Biofiltration pretreatment for reverse osmosis (RO) membrane in a water reclamation system., *Chemosphere 59*, 127-33.

37. Kim, D., Jung, S., Sohn, J., Kim, H., and Lee, S. (2009) Biocide application for controlling biofouling of SWRO membranes — an overview, *Desalination*, Elsevier B.V. *238*, 43-52.

38. Tyrovola, K., and Diamadopoulos, E. (2005) Bromate formation during ozonation of groundwater in coastal areas in Greece, *Desalination 176*, 201-209.

39. Bereschenko, L. a, Prummel, H., Euverink, G. J. W., Stams, a J. M., and Loosdrecht, M. C. M. van. (2011) Effect of conventional chemical treatment on the microbial population in a biofouling layer of reverse osmosis systems., *Water research*, Elsevier Ltd *45*, 405-16.

40. Johansen, C., Falholt, P., and Gram, L. (1997) Enzymatic removal and disinfection of bacterial biofilms., *Applied and environmental microbiology 63*, 3724-8.

41. Flemming, H.-C. (2002) Biofouling in water systems--cases, causes and countermeasures., *Applied microbiology and biotechnology 59*, 629-40.

42. Bacchin, P., Aimar, P., and Field, R. (2006) Critical and sustainable fluxes: Theory, experiments and applications, *Journal of Membrane Science 281*, 42-69.

43. Field, R., Wu, D., Howell, J. A., and Gupta, B. B. (1995) Critical flux concept for microfiltration fouling, *Journal of Membrane Science 100*, 259-272.

44. Vrouwenvelder, J., Vanpaassen, J., Vanagtmaal, J., Vanloosdrecht, M., and Kruithof, J. (2009) A critical flux to avoid biofouling of spiral wound nanofiltration and reverse osmosis membranes: Fact or fiction?, *Journal of Membrane Science 326*, 36-44.

45. Kumar, M., Adham, S. S., and Pearce, W. R. (2006) Investigation of seawater reverse osmosis fouling and its relationship to pretreatment type., *Environmental science & technology 40*, 2037-44.

46. Brink, P. van den, Zwijnenburg, A., Smith, G., Temmink, H., and Loosdrecht, M. van. (2009) Effect of free calcium concentration and ionic strength on alginate fouling in cross-flow membrane filtration, *Journal of Membrane Science 345*, 207-216.

47. Jin, X., Huang, X., and Hoek, E. M. V. (2009) Role of specific ion interactions in seawater RO membrane fouling by alginic acid., *Environmental science & technology 43*, 3580-7.

48. Tang, C. Y., Kwon, Y.-N., and Leckie, J. O. (2009) Effect of membrane chemistry and coating layer on physiochemical properties of thin film composite

polyamide RO and NF membranesII. Membrane physiochemical properties and their dependence on polyamide and coating layers, *Desalination*, Elsevier B.V. *242*, 168-182.

49. Knoell, T., Safarik, J., Cormack, T., Riley, R., Lin, S. W., and Ridgeway, H. (1999) Biofouling potentials of microporous polysulfone membranes containing a sulfonated polyether-ethersulfone/polyethersulfone block copolymer: correlation of membrane surface properties with bacterial attachment, *Journal of membrane science*, Elsevier *157*, 117-138.

50. Lee, W., Ahn, C. H., Hong, S., Kim, S., Lee, S., Baek, Y., and Yoon, J. (2010) Evaluation of surface properties of reverse osmosis membranes on the initial biofouling stages under no filtration condition, *Journal of Membrane Science*, Elsevier B.V. *351*, 112-122.

51. Liu, C. X., Zhang, D. R., He, Y., Zhao, X. S., and Bai, R. (2010) Modification of membrane surface for anti-biofouling performance: Effect of anti-adhesion and anti-bacteria approaches, *Journal of Membrane Science 346*, 121-130.

52. Li, Q., Xu, Z., and Pinnau, I. (2007) Fouling of reverse osmosis membranes by biopolymers in wastewater secondary effluent: Role of membrane surface properties and initial permeate flux, *Journal of Membrane Science 290*, 173-181.

53. Subramani, a, and Hoek, E. (2008) Direct observation of initial microbial deposition onto reverse osmosis and nanofiltration membranes, *Journal of Membrane Science 319*, 111-125.

54. Subramani, A., Huang, X., and Hoek, E. M. V. (2009) Direct observation of bacterial deposition onto clean and organic-fouled polyamide membranes., *Journal of colloid and interface science*, Elsevier Inc. *336*, 13-20.

55. Vrijenhoek, E. (2001) Influence of membrane surface properties on initial rate of colloidal fouling of reverse osmosis and nanofiltration membranes, *Journal of Membrane Science 188*, 115-128.

56. Gilron, J., Belfer, S., V\"ais\"anen, P., and Nyström, M. (2001) Effects of surface modification on antifouling and performance properties of reverse osmosis membranes, *Desalination*, Elsevier *140*, 167–179.

57. Freger, V., Gilron, J., and Belfer, S. (2002) TFC polyamide membranes modified by grafting of hydrophilic polymers: an FT-IR/AFM/TEM study, *Journal of Membrane Science*, Elsevier *209*, 283–292.

58. Herzberg, M., Sweity, A., Brami, M., Kaufman, Y., Freger, V., Oron, G., Belfer, S., and Kasher, R. (2011) Surface properties and reduced biofouling of graft-copolymers that possess oppositely charged groups., *Biomacromolecules 12*, 1169-77.

59. Li, Q., Mahendra, S., Lyon, D. Y., Brunet, L., Liga, M. V., Li, D., and Alvarez, P. J. J. (2008) Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications., *Water research*, Elsevier Ltd *42*, 4591-602.

60. Yang, H.-L., Lin, J. C.-T., and Huang, C. (2009) Application of nanosilver surface modification to RO membrane and spacer for mitigating biofouling in seawater desalination., *Water research*, Elsevier Ltd *43*, 3777-86.

61. Lee, S. Y., Kim, H. J., Patel, R., Im, S. J., Kim, J. H., and Min, B. R. (2007) Silver nanoparticles immobilized on thin film composite polyamide membrane: characterization, nanofiltration, antifouling properties, *Polymers for Advanced Technologies*, Wiley Online Library *18*, 562–568.

62. Zhu, X., Bai, R., Wee, K.-H., Liu, C., and Tang, S.-L. (2010) Membrane surfaces immobilized with ionic or reduced silver and their anti-biofouling performances, *Journal of Membrane Science*, Elsevier B.V. *363*, 278-286.

63. Zodrow, K., Brunet, L., Mahendra, S., Li, D., Zhang, A., Li, Q., and Alvarez, P. J. J. (2009) Polysulfone ultrafiltration membranes impregnated with silver nanoparticles show improved biofouling resistance and virus removal., *Water research 43*, 715-23.

64. KWAK, S.-Y., and Kim HO, S. (2001) Hybrid Organic / Inorganic Reverse Osmosis (RO) Membrane for Preparation and Characterization of TiO 2 Nanoparticle

Self-Assembled Aromatic Polyamide Membrane, *Environmental Science & Technology 35*, 2388-2394.

65. Kim, S. H., Kwak, S.-Y., Sohn, B.-H., and Park, T. H. (2003) Design of TiO2 nanoparticle self-assembled aromatic polyamide thin-film-composite (TFC) membrane as an approach to solve biofouling problem, *Journal of Membrane Science 211*, 157-165.

66. Davies, D. G. (1998) The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm, *Science 280*, 295-298.

67. Pierson, L. S., Keppenne, V. D., and Wood, D. W. (1994) Phenazine antibiotic biosynthesis in Pseudomonas aureofaciens 30-84 is regulated by PhzR in response to cell density., *Journal of bacteriology 176*, 3966-74.

68. Miller, M. B., and Bassler, B. L. (2001) Quorum sensing in bacteria, *Annual Reviews in Microbiology*, Annual Reviews 4139 El Camino Way, PO Box 10139, Palo Alto, CA 94303-0139, USA *55*, 165–199.

69. Xavier, K. (2003) LuxS quorum sensing: more than just a numbers game, *Current Opinion in Microbiology 6*, 191-197.

70. Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., and Greenberg, E. P. (1998) The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm, *Science 280*, 295-298.

71. Barrios, A. F. G., Zuo, R., Hashimoto, Y., Yang, L., Bentley, W. E., and Wood, T. K. (2006) Autoinducer 2 controls biofilm formation in Escherichia coli through a novel motility quorum-sensing regulator (MqsR, B3022), *Journal of bacteriology*, Am Soc Microbiol *188*, 305.

72. Hentzer, M., Riedel, K., Rasmussen, T. B., Heydorn, A., Andersen, J. B., Parsek, M. R., Rice, S. a, Eberl, L., Molin, S., Høiby, N., Kjelleberg, S., and Givskov, M. (2002) Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound., *Microbiology (Reading, England) 148*, 87-102.

73. Kim, C., Kim, J., Park, H.-Y., Park, H.-J., Lee, J. H., Kim, C. K., and Yoon, J. (2008) Furanone derivatives as quorum-sensing antagonists of Pseudomonas aeruginosa., *Applied microbiology and biotechnology 80*, 37-47.

74. Ponnusamy, K., Paul, D., and Kweon, J. H. (2009) Inhibition of Quorum Sensing Mechanism and Aeromonas hydrophila Biofilm Formation by Vanillin, *Environmental Engineering Science 26*, 1359-1363.

75. Paul, D., Kim, Y. S., Ponnusamy, K., and Kweon, J. H. (2009) Application of Quorum Quenching to Inhibit Biofilm Formation, *Environmental Engineering Science 26*, 1319-1324.

76. Barraud, N., Hassett, D. J., Hwang, S.-H., Rice, S. a, Kjelleberg, S., and Webb, J. S. (2006) Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa., *Journal of bacteriology 188*, 7344-53.

77. Wikipedia. Gram positive cell wall, *http://upload.wikimedia.org/wikipedia/commons/6/6f/Gram-positive_cellwall-schematic.png*.

78. Wikipedia. Peptidoglycan, *http://upload.wikimedia.org/wikipedia/commons/d/d7/Peptidoglycan_en.svg*.

79. Walker, S. L., Redman, J. a, and Elimelech, M. (2004) Role of Cell Surface Lipopolysaccharides in Escherichia coli K12 adhesion and transport., *Langmuir 20*, 7736-46.

80. Al-Tahhan, R. a, Sandrin, T. R., Bodour, a a, and Maier, R. M. (2000) Rhamnolipid-induced removal of lipopolysaccharide from Pseudomonas aeruginosa: effect on cell surface properties and interaction with hydrophobic substrates., *Applied and environmental microbiology 66*, 3262-8.

81. Walker, S. L., Redman, J. a, and Elimelech, M. (2004) Role of Cell Surface Lipopolysaccharides in Escherichia coli K12 adhesion and transport., *Langmuir : the ACS journal of surfaces and colloids 20*, 7736-46.

82. Wilkinson, S. G. (1983) Composition and structure of lipopolysaccharides from Pseudomonas aeruginosa, *Review of Infectious Diseases*, Oxford University Press *5*, S941-S949.

83. Wikipedia. Gram negative cell wall, *http://upload.wikimedia.org/wikipedia/commons/8/8b/Gram_negative_cell_wall.svg.*

84. Sigma-Aldrich. LPS structure, *http://www.sigmaaldrich.com/etc/medialib/life-science/molecular-biology/migrationmolbio1/Endo_Free_Fig1.Par.0001.Image.400.gif.*

85. Höltje, J. V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli., *Microbiology and molecular biology reviews : MMBR 62*, 181-203.

86. Lam, H., Oh, D.-C., Cava, F., Takacs, C. N., Clardy, J., Pedro, M. a de, and Waldor, M. K. (2009) D-amino acids govern stationary phase cell wall remodeling in bacteria-Supporting Material, *Science (New York, N.Y.) 325*, 1552-5.

87. Caparrós, M., Pisabarro, a G., and Pedro, M. a de. (1992) Effect of D-amino acids on structure and synthesis of peptidoglycan in Escherichia coli., *Journal of bacteriology 174*, 5549-59.

88. Zodrow, K., Brunet, L., Mahendra, S., Li, D., Zhang, A., Li, Q., and Alvarez, P. J. J. (2009) Polysulfone ultrafiltration membranes impregnated with silver nanoparticles show improved biofouling resistance and virus removal., *Water research 43*, 715-23.

89. Pang, C. M., Hong, P., Guo, H., and Liu, W.-T. (2005) Biofilm formation characteristics of bacterial isolates retrieved from a reverse osmosis membrane., *Environmental science & technology 39*, 7541-50.

90. Bereschenko, L. a, Heilig, G. H. J., Nederlof, M. M., Loosdrecht, M. C. M. van, Stams, a J. M., and Euverink, G. J. W. (2008) Molecular characterization of the bacterial communities in the different compartments of a full-scale reverse-osmosis water purification plant., *Applied and environmental microbiology 74*, 5297-304.

91. Zhang, M., Jiang, S., Tanuwidjaja, D., Voutchkov, N., Hoek, E. M. V., and Cai, B. (2011) Composition and variability of biofouling organisms in seawater reverse osmosis desalination plants., *Applied and environmental microbiology* 77, 4390-8.